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<b>14. ABSTRACT</b>  There is increasing evidence that breast cancers are driven by a small subcomponent that displays stem cell properties. We hypothesize that these breast cancer stem cells are resistant to chemotherapy and may contribute to tumor relapse. In order to provide evidence for this, we determined the effect of chemotherapeutic agents on breast cancer stem cell populations in primary mouse xenografts. Tumor regression induced by these chemotherapeutic agents is accompanied by an enrichment in cancer stem cells as determined by the stem cell marker CD44+ CD24- and Aldehyde dehydrogenase. In order to determine the clinical relevance of these studies, we have examined expression of these markers in patients receiving neoadjuvant therapy utilizing pre- and post-treatment biopsies. In two separate studies, one completed at the University of Michigan and one in collaboration with Baylor College of Medicine, we demonstrate that tumor shrinkage from neoadjuvant chemotherapy is associated with an increase in the percent of stem cells in residual tumors. These studies provide support for the cancer stem cell hypothesis and suggest that more effective therapies against breast cancer will require the development of strategies to target and eliminate the cancer stem cell population.					
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## Introduction

The proposal BC030314, Chemoresistance of Breast Cancer Stem Cells, was based on our previous description of the isolation of tumor initiating cells from human breast cancers that have stem cell properties. These properties include the ability to self-renew as well as to differentiate into the non-tumorigenic cells which form the bulk of the tumor. The objectives of this study were to test the hypothesis that breast cancer stem cells are relatively resistant to chemotherapy compared to the differentiated cells which form the bulk of the tumor and thus may contribute to relapse following therapy. This was to be accomplished by utilizing mouse xenograft models as well as markers for stem cells in a clinical neoadjuvant chemotherapy studies. The neoadjuvant chemotherapy studies were done both at the University of Michigan and with our collaborator Dr. Jenny Chang at Baylor College of Medicine in Houston. Our results, both in xenografts models as well as in the neoadjuvant trials provide support for our hypothesis that breast cancer stem cells are relatively resistant to chemotherapy. These cells may contribute to treatment resistance and to relapse following chemotherapy treatment.

## Body

### Accomplishments

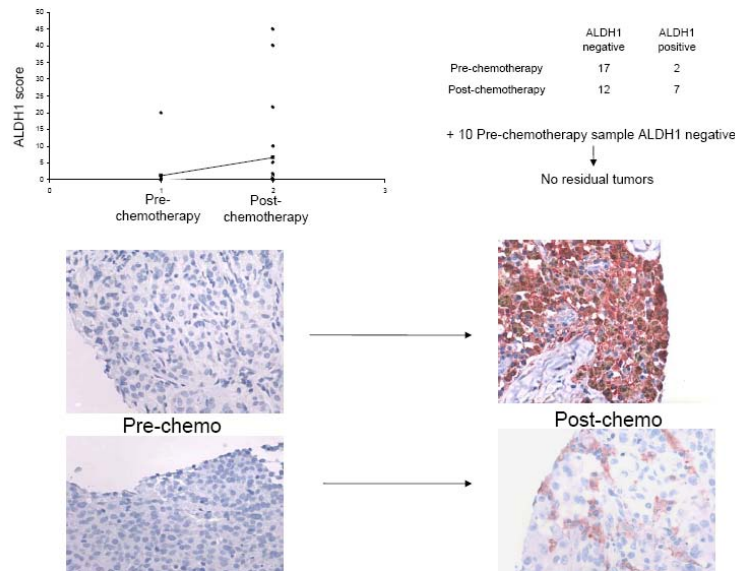
1. Creation of xenograft models-Over the course of this grant we have developed nine human tumor xenografts of tumors directly transplanted from breast cancer patients. These xenografts comprise different molecular subtypes of breast cancer including: triple negative, ER PR positive and HER2 amplified breast cancers. All of these xenografts have been successfully passaged in NOD/scid mice.
2. Validation of new stem cell marker ALDH-1-The application of stem cell biology to breast cancer research has been limited by the lack of simple assays for the identification and isolation of normal and malignant mammary stem cells. In order to complete the specific aims of this proposal, it was necessary to develop more robust and simple methods to identify breast cancer stem cells *in situ*. Utilizing *in vitro* and *in vivo* assays, we have shown that normal and malignant cells with increased Aldehyde dehydrogenase activity (ALDH) have stem/progenitor cell properties. These cells are capable of self-renewal, multilineage differentiation and are able to generate outgrowths in the humanized mammary fat pads of NOD/scid mice. In breast carcinomas, cells with high ALDH activity contain the cancer stem cell component capable of transplanting the tumor into NOD/scid mice as well as regenerating the heterogeneity of the initial tumor. In a series of 577 breast carcinomas, expression of ALDH-1 detection by immunochemistry correlated with poor clinical outcome. These findings offer a new important tool for the study of normal and malignant breast stem cells facilitating clinical application of stem cell contents [1]. ALDH is a marker of normal and malignant breast stem cells and a predictor of poor clinical outcome [1].

3. Stem cells are resistant to chemotherapy in NOD/scid mice. In order to determine the relative sensitivities and resistance of breast cancer stem cells to adriamycin and taxol, two commonly used chemotherapeutic agents, we utilized the breast cancer xenografts generated during this proposal. Utilizing these xenograft models, we demonstrated that tumor shrinkage caused by chemotherapy administration resulted in increased expression of cells expressing the stem cell markers, CD44+ CD24-. Furthermore, we have found that the expression of ALDH-1 positive cells increases following chemotherapy.
4. In order to extend these xenografts studies into the clinical setting, we have examined the effect of neoadjuvant chemotherapy on the breast cancer stem cell number. This was done both utilizing a set of neoadjuvant chemotherapy patients treated at the University of Michigan and by our collaborator at Baylor College of Medicine in Houston, Dr. Jenny Chang, utilizing neoadjuvant patients at their institution. The University of Michigan data set comprised 44 patients who received neoadjuvant chemotherapy. Breast biopsies from these patients assessed for the stem cell marker ALDH-1 expression before chemotherapy and following a course of neoadjuvant chemotherapy. Utilizing immunohistochemistry, ALDH-1 expression was detected in 14% of biopsies pre-chemotherapy biopsies but in 38% of biopsies post-chemotherapy ( $p=0.01$ , Table 1). In addition, the percent of cells expressing the stem cell marker ALDH-1 significantly increased following chemotherapy (Figure 1). These studies provided support for our hypothesis that breast cancer stem cells are relatively resistant to chemotherapy compared to cells which comprise the bulk of the tumor (manuscript in preparation).

**Table 1**

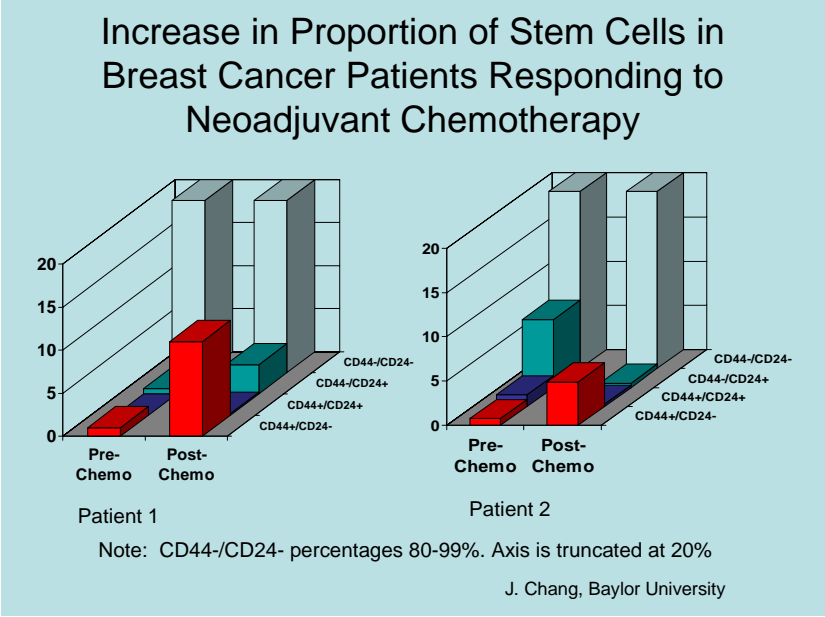
	ALDH1 negative	ALDH1 positive	
Pre-chemotherapy	38 (86%)	6 (14%)	P-value < 0.01
Post-chemotherapy	37 (62%)	23 (38%)	

**Figure 1**



In order to provide for further support for this hypothesis, we have collaborated with Dr. Jenny Chang at Baylor College of Medicine in Houston. Her group has treated 30 patients with neoadjuvant chemotherapy and measured the percent of cells expressing the stem cell marker CD44+ CD24- before and after chemotherapy. As shown in Figure 2, there was a significant increase in cells expressing stem cell markers following chemotherapy.

**Figure 2**



Thus, the results of two neoadjuvant trials utilizing two independent data sets and two different cancer stem cell marker combinations both support the hypothesis that breast cancer stem cells are relatively resistant to chemotherapy.

The relative resistance of breast cancer stem cells to chemotherapy highlights the importance of developing new approaches to target the key cell population.

Although, not originally included within the statement of work for this grant, we have thus made substantial progress in elucidating pathways which regulate stem cell behavior. These pathways include both NOTCH and Hedgehog signaling [2-7]. The role of Hedgehog signaling as well as the polycomb gene BMI-1 in regulating the self-renewal of both normal and malignant mammary stem cells was published in *Cancer Research*. Together, the results of our studies regarding the resistance of breast cancer stem cells to chemotherapy and the reliance of these cells on Hedgehog and NOTCH signaling suggests that the use of a combination of chemotherapy to target the differentiated cells and either Hedgehog or NOTCH inhibitors to target the cancer stem cell population represents a rational therapeutic strategy. Based on these studies, we have submitted a new DOD grant in collaboration with Dr. Jeff Rosen and Jenny Change at Baylor College of Medicine, to utilize these approaches to develop strategies to target the cancer stem cell population.

#### Research Accomplishments

- Generation of new xenografts obtained from breast cancer patients including ER+ and HER2/neu amplified
- Development and validation of ALDH as a marker for breast cancer stem cells.
- Use of ALDH expression to detect breast cancer stem cells *in situ* in fixed tissue specimens.
- Demonstration that taxotere and adriamycin selectively killed differentiated cells and spared the stem cell components in xenografts models.
- Demonstration that the percent of ALDH+ cells increases following neoadjuvant chemotherapy treatments in a trial done at the University of Michigan.
- Collaboration with Dr. Jenny Change at Baylor College of Medicine to demonstrate that the percent of cells expressing the stem cell marker CD44+ CD24- increases following neoadjuvant chemotherapy.
- Demonstration of the importance of Hedgehog signaling and BMI-1 in the regulation of self-renewal are both normal and breast cancer stem cells [4].
- Based on the above results, development of strategies combining chemotherapy and stem cell inhibitors for the treatment of breast cancer.

#### Conclusions

The study supported by this grant provides strong support for the cancer stem cell hypothesis. Furthermore, they support our hypothesis that breast cancer stem cells are relatively resistant to

chemotherapy. This was shown by demonstrating in human tumor xenografts in NOD/scid mice that tumor regression induced by chemotherapy was accompanied by enrichment of breast cancer stem cells. The clinical relevance of these studies were confirmed by demonstrating in two independent neoadjuvant chemotherapy studies that the percent of cells displaying stem cell markers was increased following chemotherapy. Together these studies support the hypothesis of chemoresistance of breast cancer stem cells and suggest that more effective strategies will require targeting of this cancer stem cell population.

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## Appendices:

1. Ginestier C, Hur M, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Schott A *et al*: **ALDH1 is a marker of normal and malignant breast stem cells and a predictor of poor clinical outcome.** *Cell: Stem Cell* 2007, **In press**.
2. Korkaya H, Wicha M: **Selective Targeting of Cancer Stem Cells: A New Concept in Cancer Therapeutics.** *In press*.

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Abstract: Application of stem cell biology to breast cancer research has been limited by the lack of simple assays for identification and isolation of normal and malignant stem cells. We show that stem/progenitor cells in normal breast epithelium and breast tumors have increased aldehyde dehydrogenase activity. Furthermore, immunostaining using ALDH1 antibody identifies normal and malignant stem/ progenitor cells in situ. In a series of 577 breast carcinomas on tissue microarrays, expression of ALDH1 was an independent predictor of poor prognosis. These findings provide support for the "cancer stem cell hypothesis" and offer an important new tool for the study of normal and malignant breast stem cells. Moreover, since ALDH1 immunodetection provides a simple method to identify cancer stem and progenitor cells in situ it should facilitate the clinical application of stem cell concept.

January 31, 2007

Dear Editors:

We would like to submit two separate manuscripts for publication in Cell stem cell. The first manuscript "ALDH1 is a marker of normal and breast cancer stem cells is a predictor of poor clinical outcome" and the second "Estrogen receptor expression in stem and progenitor cells defines a hierarchy of cellular differentiation in normal and malignant breast epithelium". Each of these manuscripts represents a distinct body of research; however, we are submitting them simultaneously because of the important interrelationship between these manuscripts. In particular, the second manuscript dealing with estrogen receptor expression in stem and progenitor cells relies on the data demonstrating that ALDH1 is indeed a marker of normal and malignant stem cells, which is shown in manuscript one.

In the first manuscript, we demonstrate that aldehyde dehydrogenase may be used as a marker to isolate both normal and malignant mammary stem cells. This manuscript utilizes both in vitro culture systems as well as a mouse model developed by our laboratory and represents the first description of isolation of human mammary stem cells. Furthermore, by demonstrating that this same marker is able to isolate both normal stem cells as well as cancer stem cells from human breast carcinomas, it provides strong support for the "cancer stem cell hypothesis". In a series of 577 breast carcinomas we demonstrate that the

expression of this stem cell marker is a significant independent predictor of poor prognosis. Thus, this manuscript not only provides support for the “cancer stem cell hypothesis” it offers an important new tool for the study of both normal and malignant breast stem cells.

In the second manuscript which utilizes the technology described in the first one, we examine the cellular origin of estrogen receptor expression in normal human mammary stem and progenitor cells. We demonstrate, unequivocally, that human breast stem cells are estrogen receptor negative and give rise to estrogen receptor positive progenitor cells capable of proliferation. Furthermore, we demonstrate that this hierarchy of differentiation is maintained in a subset of human breast carcinomas. This subset of ER+ cancers which is driven by an ER- cancer stem cell has a significantly worse clinical outcome than the rest of ER+ breast cancers. These studies suggest that different subtypes of estrogen receptor positive breast cancers may have different cellular origins. These studies provide a new framework for understanding estrogen regulation of mammary development and carcinogenesis. Furthermore, these findings illustrate the important application of stem cell biology to clinical material. The ability to use simple immunochemical techniques to determine co-expression of estrogen receptor and stem cell markers may permit more tailored breast cancer treatments by defining molecular subcategories of estrogen receptor positive breast cancer.

We feel that the subjects of these two manuscripts are suitable for publication in Cell stem cell since they not only define important processes in stem cell biology but demonstrate the important clinical application of these concepts.

We request that Dr Michael Clarke, Stanford University, a previous collaborator, not review these manuscripts due to potential conflicts of interest.

Thank you for your consideration.

Sincerely,

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# **ALDH1 is a marker of normal and cancer breast stem cells and a predictor of poor clinical outcome**

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**Running title:** ALDH1 in Human Breast Stem Cells

## **Abstract**

Application of stem cell biology to breast cancer research has been limited by the lack of simple assays for identification and isolation of normal and malignant stem cells. We show that stem/progenitor cells in normal breast epithelium and breast tumors have increased aldehyde dehydrogenase activity. Furthermore, immunostaining using ALDH1 antibody identifies normal and malignant stem/ progenitor cells in situ. In a series of 577 breast carcinomas on tissue microarrays, expression of ALDH1 was an independent predictor of poor prognosis. These findings provide support for the “cancer stem cell hypothesis” and offer an important new tool for the study of normal and malignant breast stem cells. Moreover, since ALDH1 immunodetection provides a simple method to identify cancer stem and progenitor cells in situ it should facilitate the clinical application of stem cell concept.

## Introduction

Although the concept that cancers arise from “stem” or “germ cells” was first proposed almost 150 years ago, it is only recently that advances in stem cell biology generated the experimental framework necessary to test this hypothesis (Reya et al., 2001; Sell et al., 2004). According to the cancer stem cell hypothesis, tumors originate in either tissue stem cells or progenitor cells through deregulation of the normally tightly regulated process of self-renewal (Molofsky et al., 2004; Passegue et al., 2003). Self-renewal is the process by which stem cells generate progeny identical to themselves. Stem cells also differentiate to generate multipotent progenitors that in turn give rise to committed progenitors and differentiated cells that ensure organ functionality. Cancer stem cells share these properties with their normal counterparts: they have self-renewal capacity driving tumorigenicity, recurrence and metastasis and they generate progeny able to differentiate, albeit aberrantly, generating a heterogeneous population of cancer cells. These differentiated cells constitute the bulk of the tumor, but they are not tumorigenic, due to their lack of self renewal capacity and limited proliferation potential. Experimental evidence supporting the cancer stem cell hypothesis was first generated in 1997 by Dicks’ group, who demonstrated that human leukemias are driven by a small population of leukemic stem cells capable of transferring the disease to NOD/scid mice (Bonnet and Dick, 1997). This concept was extended to solid tumors by Clarke and Wicha who demonstrated that human breast cancers contain a cell population with stem cell properties, characterized by the expression of the cell surface markers CD44+ CD24-lin- (Al-Hajj et al., 2003). Subsequently, cancer stem cells have been identified and prospectively isolated from a variety of malignancies, including brain cancers, prostate cancer, melanoma, multiple myeloma and colon cancer (Collins et al., 2005; Fang et al., 2005; Matsui et al., 2004; O’Brien et al., 2006; Ricci-Vitiani et al., 2006; Singh et al., 2004a; Singh et al., 2004b).

It is likely that cancer stem cells have a phenotype defined by the cell of origin (stem cells or early progenitor cells) and by the oncogenic events that contributed to transformation. Recent studies have provided evidence that supports this concept (Jamieson et al., 2004; Kelly et al., 2002). One approach for finding shared stem cell markers is to focus on conserved stem and progenitor cell functions. These functional markers may be inherited by the malignant stem cell compartment, across multiple histologic subtypes of cancer from the same tissue of origin. A candidate marker which

fits this description is aldehyde dehydrogenase 1, a detoxifying enzyme responsible for the oxidation of intracellular aldehydes (Duester, 2000; Magni et al., 1996; Sophos and Vasiliou, 2003; Yoshida et al., 1998). ALDH may have a role in early differentiation of stem cells, through its role in oxidizing retinol to retinoic acid (Chute et al., 2006). It has been shown that murine and human hematopoietic and neural stem and progenitor cells have a high ALDH activity (Armstrong et al., 2004; Hess et al., 2004; Hess et al., 2006; Matsui et al., 2004). Increased ALDH activity has also been found in stem cell populations in multiple myeloma and acute myeloid leukemia (AML) (Matsui et al., 2004; Pearce et al., 2005). Aldehyde dehydrogenase activity may thus provide a common marker for both normal and malignant stem and progenitor cells.

In the present study we demonstrate that cells with ALDH activity isolated from normal human breast have phenotypic and functional characteristics of mammary stem cells. Moreover, the ALDH<sup>+</sup> cells isolated from human breast tumors contain the cancer stem cell population. We also demonstrate that both normal and malignant human mammary stem cells may be identified in situ in breast carcinoma specimens. Analyzing the expression of ALDH1 in 577 human breast carcinomas from two patient populations, we show that the expression of this stem/progenitor cell marker is a powerful predictor of poor clinical outcome. These findings provide support for the “cancer stem cell hypothesis” and open new possibilities for the study of mammary stem/progenitor cells and their role in mammary development and carcinogenesis. In addition, ALDH1 immunodetection is a simple method for identifying cancer stem/progenitor cells in situ, facilitating the clinical application of stem cell concepts.

## Results

**The ALDEFLUOR-positive population isolated from normal mammary epithelium has stem cell properties.** Single cell suspensions of normal mammary epithelial cells were obtained by mechanical and enzymatic digestion of breast reduction samples, as previously described (Dontu et al., 2003). We utilized the ALDEFLUOR assay (Stem Cell Technologies) to assess the presence and size of the population with ALDH enzymatic activity in normal human breast epithelium. Analysis of breast reduction samples from 14 different patients showed an average of 8% ( $8.18 \pm 4.31$ ,  $n=14$ ) ALDEFLUOR-positive population in normal mammary epithelial cells (**Figure 1, A and B**).

Using previously established in vitro and in vivo assays (Dontu et al., 2003; Kuperwasser et al., 2004; Stingl et al., 2006) we now show that functional characteristics associated with adult stem cells are displayed by the ALDEFLUOR-positive but not the ALDEFLUOR-negative population.

The ALDEFLUOR-positive population (**Figure 1C**), but not the ALDEFLUOR-negative population (**Figure 1D**) was capable of generating mammospheres in suspension culture. We have previously shown that mammary epithelial cells that survive and proliferate in anchorage-independent conditions are likely to be breast stem cells with self-renewal capacity (Dontu et al., 2003).

In a clonogenic assay that assesses the lineage differentiation potential of single cells, the ALDEFLUOR-positive cells were enriched in bi-lineage progenitor cells that generated mixed ESA+CD10+ colonies (**Figure 1E-H**). These represented  $67.2 \pm 3.5\%$  of the total number of colonies formed, whereas for the ALDEFLUOR-negative cells they represented only  $9.1 \pm 1.3\%$ , (**Figure 1E-H**).

Differentiation potential of ALDEFLUOR-positive and -negative populations was also assessed by flow cytometry analysis of lineage-specific markers expressed in the progeny of these cells, generated in cultivation conditions that promote differentiation. The results confirmed the findings of the clonogenic assay (**Figure 1I**). The ALDEFLUOR-positive population is enriched in progenitors cells, which generate bi-potent progeny ( $15.3 \pm 3.2\%$ , CD10-/ESA-;  $21.2 \pm 1.5\%$ , CD10+/ESA+), myoepithelial ( $2.1 \pm 0.3\%$ , CD10+/ESA-) and luminal epithelial cells ( $63.2 \pm 4.1\%$ , CD10-/ESA+)

(**Figure 1I**, *left panel*). The ALDEFLUOR-negative population contains progenitors restricted to the luminal epithelial cell fate ( $93.5 \pm 3.4\%$ , CD10-/ESA+) (**Figure 1I**, *right panel*).

We utilized the mouse model described by Kuperwasser et al. to evaluate the ability of sorted cells from normal breast epithelium to grow and differentiate in vivo (Kuperwasser et al., 2004). ALDEFLUOR-positive, ALDEFLUOR-negative and unsorted cells were transplanted into humanized cleared mammary fat pads of NOD/scid mice (25,000, 5,000, and 500 ALDEFLUOR-positive cells, 50,000, 5,000 and 500 ALDEFLUOR-negative cells, and 25,000, 5,000, and 500 unsorted cells). Only ALDEFLUOR-positive and unsorted cells had outgrowth potential (**Supplementary Table1**) as shown by ducts formation upon implantation of 25,000 cells (**Figure 1J**). As is the case in the human mammary tree, these small ducts were composed of a luminal epithelial layer, expressing CK18 (**Figure 1L**) and an outer myoepithelial cell layer, expressing smooth muscle actin (SMA) (**Figure 1M**). The ALDEFLUOR-negative population failed to repopulate the fat pads, even when 50,000 cells were injected (**Figure 1K**).

Taken together, the results of the in vivo and in vitro assays indicate that the Aldefluor-positive cells represent the cell population with the broadest differentiation potential and highest ability to grow in vivo.

**In situ characterization of ALDH1-positive cells in normal breast epithelium and mammosphere sections.** We next determined whether ALDH1 immunohistochemistry (IHC) could be utilized to detect mammary stem/progenitor cells in situ. We utilized flow cytometry analysis to determine the overlap between the cell population with a high ALDH enzymatic activity (ALDEFLUOR-positive) and the population immunostained by ALDH1. The ALDEFLUOR-positive and -negative populations from normal breast epithelium were isolated by FACS, fixed, and stained with an ALDH1 monoclonal antibody. The cells detected by immunostaining are contained in the ALDEFLUOR-positive population, whereas the ALDEFLUOR-negative population contains no ALDH1-positive cells. (**Supplementary Figure1**)

Immunostaining of paraffin-embedded sections of normal breast epithelium using the ALDH1 antibody identified a relatively rare population of ALDH1-positive cells located in

the terminal ductal lobular units (TDLUs). ALDH1-positive cells appeared to form a bridge in the lumen that could be located at the bifurcation point of side branches in the TDLUs (**Figure 2A**). A stem cell marker should not colocalize with markers of mature differentiated mammary epithelial cells. We performed double staining with ALDH1 and CK18, a marker of luminal epithelial cells and ALDH1 and SMA, a marker of myoepithelial cells. The ALDH1-positive cells did not co-localize with CK18, or SMA in sections through normal human breast epithelium (**Figure 2C-D**). This indicated that ALDH1-positive cells are not mature, differentiated luminal epithelial or myoepithelial cells. Although the phenotype of normal stem and/or progenitor cells from the human breast epithelium has not been identified, several markers including CK5/6 have been found expressed in undifferentiated mammary epithelial cells. We did not detect overlapping expression between CK5/6 and ALDH1 in sections through normal human breast epithelium (data not shown). To determine if this resulted from the scarcity of these two populations, we repeated the same analysis on mammosphere sections. We have shown previously that the mammospheres generated from normal mammary epithelium are enriched in stem/progenitor cells (Dontu et al., 2003). Immunostaining of mammosphere sections using ALDH1 antibody showed that the ALDH1-positive cells are present in small numbers in the mammospheres, comprising approx 5% of the total population (**Figure 2B**). A subset of these ALDH1-positive cells express CK5/6 (**Figure 2E**). These results are consistent with the hypothesis that ALDH1-positive cells represent the stem/progenitor population of the normal human breast epithelium.

**ALDEFLUOR-positive breast carcinoma cells display properties of cancer stem cells.** To investigate the tumorigenicity of the ALDEFLUOR-positive population in breast cancers we established xenotransplants from four independent human breast cancers (MC1, UM1, UM2, UM3). Cells from these tumors were transplanted orthotopically in the humanized cleared fat-pad of NOD/scid mice, without cultivation in vitro. The tumors were human invasive ductal carcinomas, three ER-PR-ERBB2- (MC1, UM1, and UM3) and one ER+PR+ERBB2- (UM2). The tumorigenicity of the sorted ALDEFLUOR populations was assessed in early passages in the animals. In contrast to assays that test tumorigenicity of sorted populations directly from patient tumors, this experimental design minimizes the bias introduced by the variable ability of breast cancers to xenotransplant. We found that the ALDEFLUOR-positive population in these three

tumors constituted between 3% to 10% of the total cell population (**Figure 3A-B and Supplementary Figure 2**). We performed serial passages using, limiting dilutions of ALDEFLUOR-positive, -negative and unsorted cells (50,000 cells; 25,000 cells; 5,000 cells; 500 cells) in the humanized cleared fat pad of NOD/scid mice. For each of the three tumors and for each of the three passages performed, only the ALDEFLUOR-positive population formed tumors, even when implanted in low numbers (**Table 1 and Supplementary Figure 2**). As shown in **Figure 3D**, tumor size and latency of tumor formation correlated with the number of ALDEFLUOR-positive cells injected. Remarkably, 500 ALDEFLUOR-positive cells generated a tumor in as few as 40 days. ALDEFLUOR-negative cells failed to reproducibly generate tumors although a limited growth was produced when 50,000 ALDEFLUOR-negative cells were injected. This is consistent with the presence of less than 0.01% contaminating ALDEFLUOR-positive cells, which is within the limits of FACS error (**Figure 3E**). H&E staining of the fat pad sections confirmed that tumors formed by ALDEFLUOR-positive cells contained malignant cells (**Figure 3F**) whereas only residual Matrigel, apoptotic cells and mouse tissue was seen at the sites of the ALDEFLUOR-negative cell injections (**Figure 3G**). No tumors were detected at these sites after 20–34 weeks.

Consistent with the ALDEFLUOR-positive population having stem cell characteristics, tumors generated by this population recapitulated the phenotypic heterogeneity of the initial tumor with a similar ratio of ALDEFLUOR-positive and negative cells (**Figure 3C**). This indicates that the ALDEFLUOR-positive cells were able to self-renew, generating ALDEFLUOR-positive cells and were able to differentiate, generating ALDEFLUOR-negative cells. These results indicate that the cancer stem/progenitor cell population in these tumors has a high ALDH enzymatic activity and may be isolated by the ALDEFLUOR assay.

**Analysis of ALDH1 protein on tissue microarrays (TMA) and correlation with histoclinical parameters.** To assess the potential use of ALDH1 expression as a diagnostic and prognostic marker in breast cancer, we analyzed expression of ALDH1 in two independent sets of breast tumors (U.M. set, I.P.C. set), by IHC on tissue microarrays (TMAs). Among these two sets, 481 tumors were available for ALDH1 staining, with 136 cases from the U.M. set and 345 cases from the I.P.C. set. In the U.M. set, 24 tumors (19%) expressed ALDH1 and 122 tumors (81%) did not. Similar results

were obtained in the I.P.C. set with 102 cases (30%) positive for ALDH1 staining and 243 cases negative (70%) (**Figure 4A-D**). Consistent with the idea that cancer stem cells represent a minority of the tumor population, ALDH1-positive cells represented an average of 5% of cells in tumors expressing ALDH1. Only two of the 481 tumors had ALDH1 staining in the vast majority of the cell population (**Figure 4A**). We next determined whether ALDH1 expression correlates with the histoclinical characteristics of the breast cancers. We found similar results in both sets (**Table 2**). ALDH1-positive tumors were associated with high histological grade ( $p<0.05$  ; U.M. set,  $p<0.001$  ; I.P.C. set, Fisher's exact test), ERBB2 overexpression ( $p<0.05$  ; U.M. set,  $p<0.001$  ; I.P.C. set, Fisher's exact test) and absence of estrogen and progesterone receptor expression ( $p<0.05$  ; U.M. set,  $p<0.0001$  ; I.P.C. set, Fisher's exact test). No correlation was found with age, tumor size, and lymph node metastasis.

**ALDH1 protein expression and clinical outcome.** Analysis of overall survival (OS) showed a strong association of ALDH1-positive tumors with poor clinical outcome for both populations ( $p=0.0459$  ; U.M. set,  $p=0.000675$  ; I.P.C. set, log-Rank test) (**Figure 4E-F**). In the U.M. set, the 5-year OS was 19.8% [14.52-97.28] for patients with an ALDH1-positive tumor and 58.7% [33.22-100] for patients with an ALDH1-negative tumor. In the I.P.C. set, the 5-year OS was 69.59% [60.73-79.73] for patients with an ALDH1-positive tumor and 84.55% [80.02-89.33] for patients with an ALDH1-negative tumor.

We performed a Cox multivariate analysis of OS in which the values for ALDH1, tumor size, age, lymph node metastasis, histological grade, ER, PR, Ki-67 and ERBB2 were considered as categorical variables. ALDH1 expression was an independent prognostic factor as was Ki-67 status, tumor size, and histological grade (**Figure 4-G**). The relative risk of death due to cancer was 1.76 for patients with ALDH1-positive tumors compared to patients with ALDH1-negative tumors ( $p<0.028$ ).

## Discussion

The cancer stem cells hypothesis has fundamental implications for cancer biology in addition to clinical implications for cancer risk assessment, early detection, prognostication, and prevention. Furthermore, the development of cancer therapeutics based on tumor regression may have produced agents which kill differentiated tumor cells while sparing the small cancer stem cell population (Wicha et al., 2006). The development of more effective cancer therapies may thus require targeting this important cancer stem cell population. The success of these new approaches hinges on the identification, isolation and characterization of cancer stem cells. Recently, the phenotype of the mouse mammary stem cells was identified by several groups (Shackelton et al., 2006; Stingl et al., 2006). These studies showed that an entire, functional mammary gland can be regenerated in vivo in several serial passages, starting from a single cell (Shackelton et al., 2006). Also, considerable progress has been made recently towards identification of human mammary stem cells, although the phenotype of these cells has remained elusive (Clarke et al., 2006). Our study indicates that ALDH1 is a marker of stem/progenitor cells of the normal human breast and breast carcinomas. Moreover, identification of normal and malignant stem/progenitor cells by the same marker supports the concept that stem and progenitor cells are primary targets of transformation, and thus lends further support to the cancer stem cell hypothesis. In addition, the ability to identify stem/progenitor cells by this shared phenotypic trait, ALDH1 expression permits analysis of carcinogenesis from normal to pre-malignant and then malignant state. Unlike the previously described breast cancer stem cell phenotype, which requires the use of a combination of ten surface antigens (Al-Hajj et al., 2003), ALDH1 provides a simple tool to identify normal and cancer stem cells. In addition, the simplicity of this technique offers an important advantage for clinical applications. We show in the present study that ALDH1 detection by immunohistochemistry on paraffin-embedded sections is a reliable method for detecting cancer stem cells *in situ*. Moreover, ALDH1 expression is a powerful prognostic factor for breast cancer and it has direct or inverse correlation with known histoclinical parameters, such as tumor grade, ER/PR status and ERBB2 overexpression.

In the vast majority of breast tumors analyzed in this study the ALDH1 positive cells represented a relatively small population, consistent with the notion that cancer stem

cells represent a minority of the tumor population. Remarkably, only two tumors out of 481 analyzed, had a predominant ALDH1 positive population. These tumors had a very aggressive clinical evolution and may have been driven by a stem cell population locked in self-renewal, undergoing little or no differentiation.

We and others have proposed that cancer stem cells by virtue of resistance to current treatment modalities may contribute to tumor recurrence following therapy. Since ALDH has been shown to metabolize a number of chemotherapeutic agents such as cyclophosphamide (Moreb et al., 1992), expression of ALDH1 in breast cancer stem cells may contribute to resistance of these cells to cyclophosphamide, an agent frequently used in the treatment in breast cancer.

We propose that ALDH 1 expression in a subset of tumors may reflect transformation of ALDH1-positive stem or early progenitor cells in these tumors. By contrast, ALDH1-negative tumors may be generated by the transformation of an ALDH1-negative progenitor cells. In the ALDH1-positive tumors, the cancer stem cell population may inherit properties of normal stem cells that confer aggressiveness: ability to self-renew, high proliferation potential, resistance to damaging agents, and chemoresistance. This hypothesis is consistent with the studies of AML (Bonnet et al., 1999). Alternatively, ALDH1-negative tumors may contain rare ALDH1-positive cells below the level of detection by immunostaining on TMAs. The detection of an ALDH1-positive population in TMAs cores may be due to an increased self-renewal activity in these tumors. A recent study has shown that a gene expression signature associated with increased self-renewal of normal stem cells is a predictor of poor prognosis (Glinsky et al., 2005; Lahad et al., 2005). In agreement with our findings, a previously described molecular signatures of breast cancer associated with a poor prognosis for breast cancer contain one or more ALDH isotypes (Alexe et al., 2006). Recently, a combinatorial analysis of gene expression data was used to re-analyze the van't Veer breast cancer gene expression data set (van't Veer et al., 2002). This analysis identified 17 genes associated with poor prognosis in breast cancer, two of which were ALDH isotypes. Moreover, a recent study showed that granulocyte macrophage progenitor cells, transformed by the MLL-AF9 fusion protein, retained the global expression profile of their normal cells of origin and had only a subset of genes re-programmed. This set included 363 genes which were associated with self-renewal in normal hematopoietic stem cells including an ALDH isotype (Krivtsov et al., 2006).

In conclusion, our study lends support to the cancer stem cell hypothesis, by showing that both normal and malignant mammary stem cells share a common functional marker, ALDH1. Identification of ALDH1 as a marker of normal and malignant human breast stem cells opens important new avenues of research in normal breast development of and carcinogenesis. Furthermore, we demonstrate that ALDH1 expression may be used to detect both normal and malignant mammary stem cells in situ, in fixed paraffin-embedded sections. The clinical utility and relevance of this assay was demonstrated by a strong association of ALDH1 expression with clinical outcome in two independent tumor sets, totaling 577 patients. Since ALDH is also expressed in hematopoietic and neuronal stem cells, this marker may prove useful for the detection and isolation of cancer stem cells in other malignancies, thus facilitating the application of cancer stem cell biology to clinical practice.

## **Experimental Procedures**

### **Dissociation of normal breast epithelium**

Normal breast tissue from reduction mammoplasties was minced with scalpels and dissociated enzymatically as previously described. Fibroblasts, endothelial cells and blood cells were removed by differential centrifugation and by treatment with ammonium chloride solution. This method generates a suspension of cells highly enriched in epithelial cells (95-99% purity). To generate single cell suspension for the in vivo implantation, collagenase digestion time (6h) was shorter (Dontu et al., 2003).

The mammoplasty samples were procured and utilized according to approved IRBMED protocols for research in human subjects.

### **Mammosphere culture**

Single cells were plated in ultra-low attachment plates (Corning, Acton, MA, USA) or plates coated with 1% agarose in PBS, at a density of 100,000 viable cells/ml in primary culture and 5000 cells/ml in subsequent passages. For mammosphere culture, cells were grown in a serum-free mammary epithelial basal medium (MEBM) (Cambrex Bio Science Walkersville, Inc, Walkersville, MD, USA) supplemented with B27 (INVITROGEN, Carlsbad, CA, USA), 20 ng/mL EGF (BD Biosciences, San Jose, CA, USA), antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 ug/ml streptomycin sulfate and 0.25 µg/ml amphotericin B), 20 ug/ml Gentamycin, 1 ng/ml Hydrocortisone, 5 µg/ml Insulin and 100 µM beta-mercaptoethanol (GIBCO™ INVITROGEN) in a humidified incubator (10% CO<sub>2</sub>: 95% air, 37(C) for 7-10 days as previously described (Dontu et al., 2003).

### **Differentiating culture conditions**

Single cell suspensions were plated on collagen-coated plates at a density of 2000 viable cells/10 cm diameter dish. Cells were grown in Ham's F-12 medium (GIBCO™ INVITROGEN) with 5% fetal bovine serum (FBS), 5 µg/ml insulin, 1 µg/ml hydrocortisone, 10 µg/ml cholera toxin (Sigma, St Louis, MO, USA), 10 ng/ml epidermal growth factor (BD Biosciences) and 1X Pen/Strep/Fungizone Mix (GIBCO). Cells were fixed or collected for immunostaining after 12 days.

## **Flow cytometry**

Cells were stained fresh or after fixation in methanol. Primary antibodies: ESA labeled FITC, CD10 labeled PE (dilution 1:25, Novocastra, Newcastle, UK) and ALDH1 (dilution 1/100, BD Biosciences) were used for immunostaining. Incubation was performed for 20 min. on ice in Hanks Balanced Salt Solution (HBSS, GIBCO) with 2% FBS, followed by washing in HBSS with 2% FBS. For ALDH1 staining, the same procedure was applied. Secondary antibody used was anti-mouse IgG, labeled with PE (1:250 dilution; Jackson Labs, MA, USA). After incubation, cells were washed once with HBSS and were resuspended in HBSS supplemented with 5% FBS. Fresh cells were stained with 1 µg/ml propidium iodide (PI) (Sigma) for 5 min. for viability. Analysis was performed using FACStarPLUS (Becton Dickinson, Palo Alto, CA, USA) flow cytometer.

## **Xenotransplants samples**

Human breast tumors were obtained as biopsy cores or pieces of tumors after surgery and implanted in humanized cleared fat pads of NOD/SCID mice for establishing xenotransplants. Four xenotransplants were used: an ER-PR-ERBB2- tumor at the 15th passage in the animals (MC1), an ER-PR-ERBB2- tumor at the 3rd passage (UM1), an ER+PR+ERBB2- tumor at the 4th passage in the animals (UM2), and an ER-PR-ERBB2- tumor at the 2nd passage (UM3). Two of the xenotransplants were generated from metastatic tumors (MC1, pleural effusion and UM2, ovarian metastasis) and two from primary tumors (UM1, UM3).

## **Aldefluor assay and separation of the ALDH positive population by FACS**

The ALDEFLUOR kit (StemCell technologies, Durham, NC, USA) was used to isolate the population with a high ALDH enzymatic activity. Cells obtain from freshly dissociated normal breast epithelium or breast cancer xenografts were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 µmol/l per  $1 \times 10^6$  cells) and incubated during 40 minutes at 37°C. In each experiment a sample of cells was stained under identical conditions with 50mmol/L of specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) as negative control. Flow cytometry based sorting was conducted using a FACStarPLUS (Becton Dickinson). ALDEFLUOR fluorescence was excited at 488 nm and fluorescence emission was detected using a standard fluorescein isothiocyanate (FITC) 530/30 band pass filter. In addition, for the xenotransplanted tumors, incubation

with an anti-H2Kd antibody (BD biosciences, 1/200, 20 min on ice) followed by a secondary antibody labeled with phycoerythrin (PE) (Jackson labs, 1/250, 20 min on ice) were used to eliminate cells of mouse origin. The sorting gates were established using as negative controls the PI stained cells for viability, the ALDEFLUOR-stained cells treated with DEAB and the staining with secondary antibody alone.

### **Animal model**

NOD/SCID mice were used to assess the in vivo stem cell potential of the ALDEFLUOR-positive population, compared to the ALDEFLUOR-negative population and the unsorted population, from the normal breast epithelium and the four tumor xenografts. The animal model was described by Kuperwasser et al for xenotransplantation of normal mammary epithelial cells (Kuperwasser et al., 2004). The fat pads were cleared pre-puberty and humanized by injecting a mixture of irradiated and non-irradiated immortalized human fibroblasts (1:1 irradiated:non-irradiated, 50,000 cells/100µl Matrigel/fat pad). Irradiated fibroblasts (4Gy) support growth of normal and cancer epithelial cells by secreting a variety of growth factors, collagen and possibly directly interacting with the epithelial cells (Orimo et al., 2005; Tlsty et al., 2001). The immortalized fibroblasts were primary human mammary fibroblasts stably transfected with a retrovirus construct expressing telomerase. The fibroblast cell line is a generous gift from Dr. John Stingl and Dr. Connie Eaves (Terry Fox Laboratory, Vancouver, British Columbia, Canada). Estrogen pellets were implanted subcutaneously at the time of the clearing. The human normal breast or cancer cells were mixed with Matrigel (BD biosciences) (1:1) and implanted in the cleared humanized fat pads 2-4 weeks later. The animals injected with normal breast cells were euthanized after 10 weeks. The animals injected with cancer were euthanized when the tumors were approximately 1.2 cm in the largest diameter, to avoid tumor necrosis and in compliance with regulations for use of vertebrate animal in research. A portion of each fat pad injected was fixed in formalin and embedded in paraffin for histological analysis.

The animal studies were approved by the ULAM committee for research invertebrate animals.

### **Tissue Microarrays**

The TMAs were provided by the Tissue Microarray Core laboratory at University of Michigan Medical School and by the Laboratoire d'Oncologie Moléculaire, Institut Paoli-Calmettes de Marseille. The first TMA contained 154 breast cancer cores from a consecutive population of patients treated at the University of Michigan Hospital, MI, USA (U.M. set) between 1984 and 1991 and the second TMA contained 552 breast cancer cores from a consecutive population of patients treated at the Institut Paoli-Calmettes, Marseille, France (I.P.C. set) between 1987 and 1999. Clinical and histopathological data are available for these patients (Jacquemier et al., 2005; Kleer et al., 2003).

### **Immunostaining**

To assess the lineage composition of the colonies, cells were fixed on plates for 20 min in methanol, at -20°C, and were then stained using Peroxidase Histostain-Plus and Alkaline-phosphatase Histostain-Plus kits (Zymed, South San Francisco, CA, USA), according to the manufacturer's protocol. The primary antibodies, epithelial-specific antigen (ESA) for luminal epithelial cells and CD10 for myoepithelial cells, were used at the dilutions indicated by the manufacturer. DAB (Zymed) was used for ESA staining as substrate for peroxidase, and NBT/BCIP (Gibco) was used for CD10 staining as substrates for alkaline phosphatase.

For ALDH1 immunostaining, the paraffin-embedded sections through mammospheres, normal breast tissue and the TMA were deparaffinized in xylene and rehydrated in graded alcohol. Antigen enhancement was done by incubating the sections in citrate buffer pH6 (Dakocytomation, Copenhagen, Denmark) as recommended. Staining was done using Peroxidase histostain-Plus Kit (Zymed) according to the manufacturer's protocol. ALDH1 antibody (BD biosciences) were used at a 1/100 dilution and incubated for 1 hour. AEC (Zymed) was used as substrate for peroxidase. Slides were counter-stained with hematoxylin, and coverslipped using glycerin. TMA results were expressed in terms of percentage (P) and intensity (I) of positive cells as described previously (Ginestier et al., 2002). Results were scored by the quick score (Q) ( $Q = P \times I$ ). For the TMA, the mean of the score of minimum 2 core biopsies was calculated for each case.

For fluorescent double staining, the primary antibodies cytokeratin 18, smooth muscle actin (SMA), and cytokeratin 5/6 (Novocastra) were used at the dilutions indicated by

the manufacturer and incubated 1 hour at room temperature. Texas-red and FITC labeled secondary antibodies (Jackson Labs) were used at the dilution 1/250 and incubated for 20 minutes. Nuclei were counterstained with DAPI/antifade (INVITROGEN) and coverslipped. Sections were examined with a fluorescent microscope (Leica, Bannockborn, IL, USA).

## **Statistical analysis**

Distributions of molecular markers and other categorical variables were compared using standard  $\chi^2$  tests or Fisher exact test. The overall survival interval was calculated from the date of diagnosis. For graphical presentation, follow-up was truncated at 100 months. Survival curves were derived from Kaplan-Meier estimates and the curves were compared by logrank tests. The influence of ALDH1 expression status was assessed in multivariate analysis by the Cox proportional hazard models with a stepwise selection. The model was adjusted for usual prognostic or predictive factors in breast cancer, including tumor size, age, lymph node metastasis, histological grade, ER, PR, Ki-67 and ERBB2 status. All statistical tests were 2-sided at the 5% level of significance, and were done using the R Version 2.3.0 software. Survival rates and relative risks (RR) are presented with their 95% confidence intervals (CI).

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## Tables

**Table 1. Tumorigenicity in the humanized fat pad of NOD/SCID mice.**

	Tumors/injections			
	5x10 <sup>4</sup>	2.5x10 <sup>4</sup>	5x10 <sup>3</sup>	500
<b>Mouse Passage 1</b>				
ALDEFLUOR-negative	3/6	0/1	0/6	0/6
ALDEFLUOR-positive	5/5	1/1	4/4	6/6
Unsorted	5/5	1/1	4/4	3/4
<b>Mouse Passage 2</b>				
ALDEFLUOR-negative	1/4	---	---	0/4
ALDEFLUOR-positive	4/4	---	---	4/4
Unsorted	4/4	---	---	4/4
<b>Mouse Passage 3</b>				
ALDEFLUOR-negative	0/4	---	---	0/4
ALDEFLUOR-positive	4/4	---	---	4/4
Unsorted	4/4	---	---	4/4

**Table 2. Correlation between ALDH1 protein expression and histoclinical characteristics**

Characteristics	U.M. set					I.P.C. set				
	ALDH1 Negative		ALDH1 Positive		p-value	ALDH1 Negative		ALDH1 Positive		p-value
	No. of patients	(%)	No. of patients	(%)		No. of patients	(%)	No. of patients	(%)	
<b>All cases</b>	122	(81)	24	(19)		243	(70)	102	(30)	
<b>Age (years)</b>										
≤50	33	(27)	6	(25)	NS	79	(35)	25	(25)	NS
>50	89	(73)	18	(75)		164	(65)	77	(75)	
<b>Pathological tumor size</b>										
PT1	61	(60)	10	(45)	NS	103	(42)	37	(37)	NS
PT2	33	(33)	7	(32)		108	(45)	46	(47)	
PT3	7	(7)	5	(23)		30	(13)	16	(16)	
<b>SBR grade</b>										
I	25	(22)	2	(9)	<0.05	80	(33)	24	(24)	<0.001
II	55	(49)	7	(30)		120	(49)	38	(38)	
III	33	(29)	14	(61)		43	(18)	39	(38)	
<b>Lymph node metastasis</b>										
Negative	53	(56)	9	(41)	NS	134	(56)	43	(44)	NS
Positive	42	(44)	13	(59)		107	(44)	55	(56)	
<b>Estrogen receptor</b>										
Negative	38	(34)	14	(61)	<0.05	41	(17)	39	(39)	<0.0001
Positive	75	(66)	10	(39)		198	(83)	61	(61)	
<b>Progesterone receptor</b>										
Negative	50	(44)	15	(62)	<0.05	61	(26)	51	(52)	<0.0001
Positive	64	(56)	9	(38)		170	(74)	47	(48)	
<b>Ki-67 status</b>										
Negative (<20)	---	---	---	---	---	193	(91)	77	(82)	<0.05
Positive (≥20)	---	---	---	---	---	19	(9)	17	(18)	
<b>ERBB2 receptor</b>										
Negative (0/1+)	94	(83)	15	(62)	<0.05	208	(94)	76	(79)	<0.001
Positive (2+/3+)	19	(17)	9	(38)		14	(6)	20	(21)	

## Figure legends

**Figure 1. ALDEFLUOR positive cells from normal breast epithelium have stem cell properties.** **A-B.** Representative FACS analysis of ALDH activity of normal breast epithelial cells analyzed by the ALDEFLUOR assay. Cells incubated with ALDEFLUOR substrate (BAAA) and the specific inhibitor of ALDH, DEAB, were used to establish the baseline fluorescence of these cells (R1) and to define the ALDEFLUOR-positive region (R2) (**A**). Incubation of cells with ALDEFLUOR substrate in the absence of DEAB induces a shift in BAAA fluorescence defining the ALDEFLUOR-positive population (**B**). **C-D.** ALDEFLUOR-positive cells sorted from fresh reduction mammoplasties generated mammospheres in suspension culture (**C**), whereas ALDEFLUOR-negative cells did not (**D**). **E-H.** Evaluation of the differentiation potential of ALDEFLUOR-positive and ALDEFLUOR-negative cells. Sorted cells were grown in differentiating conditions for 12 days and stained by IHC with lineage-specific markers (ESA, CD10). The ALDEFLUOR-positive population generated 67.2  $\pm$  3.5% bi-potent progeny (ESA+ cells stained in brown and CD10+ stained in purple) (**F**), 2.9  $\pm$  0.5% myoepithelial colonies (CD10+) (**G**), and 30.6  $\pm$  5.4% luminal colonies (ESA+) (**H**), whereas the ALDEFLUOR-negative population produced 90.8  $\pm$  3.1% luminal epithelial colonies (ESA+) (**H**), and only 9.1  $\pm$  1.3% bi-potent progeny (**E**). **I.** ALDEFLUOR-positive and ALDEFLUOR-negative cells grown in differentiating conditions were collected for flow cytometry analysis of lineage markers (ESA, CD10). ALDEFLUOR-positive cells generated bi-potent progeny (15.3  $\pm$  3.2%, CD10-/ESA-; 21.2  $\pm$  1.5%, CD10+/ESA+), luminal cells (63.2  $\pm$  4.1%, CD10-/ESA+) and myoepithelial cells (2.1  $\pm$  0.3%, CD10+/ESA-) whereas ALDEFLUOR-negative cells generated predominantly luminal cells (93.5  $\pm$  3.4%, CD10-/ESA+). **J-M.** Small ducts produced in the NOD/scid humanized cleared fat pads injected with ALDEFLUOR-positive cells from normal breast epithelium. Hematoxylin and eosin staining revealing the presence of several small ducts at the site of injected ALDEFLUOR-positive cells (**J**) and residual Matrigel and mouse tissue at the site injected with ALDEFLUOR-negative cells (**K**). Immunostaining of the ducts produced by ALDEFLUOR-positive cells showed the presence of a luminal layer (stained with anti-CK18 brown staining) (**L**) and a myoepithelial layer (stained with SMA, red staining) (**M**).

**Figure 2. Characterization of ALDH1 positive-cells in the normal breast epithelium and mammosphere section.** **A.** ALDH1 staining of normal breast epithelium. ALDH1-positive cells (red cytoplasmic staining) were in a luminal position, bridging across the lumen, probably at branching points of side-ducts (arrow). **B.** ALDH1 staining in mammospheres. Only 1-5 cells/mammosphere showed positive staining for ALDH1, (approximately 5% of the total population). **C-D.** Immunofluorescence of normal breast epithelium. **C.** Double staining with CK18 (red) and ALDH1 (green). Composite image (merge) showed absence of overlap between CK18 positive cells (mature luminal cells) and ALDH1-positive cells (arrow). **D.** Double staining with SMA (green) and ALDH1 (red). Composite image (merge) showed absence of overlap between SMA positive cells (mature myoepithelial cells) and ALDH1-positive cells (arrow). **E.** Immunofluorescence of mammosphere sections. Double staining with CK5/6 (green) and ALDH1 (red). Composite image (merge) showed that only few ALDH1-positive cells displayed an exclusive red signal (arrow) whereas all the CK5/6 positive cells (asterisk) displayed a hybrid signal (yellow) corresponding to cells positive for ALDH1 and CK5/6. All nuclei were counterstained in DAPI.

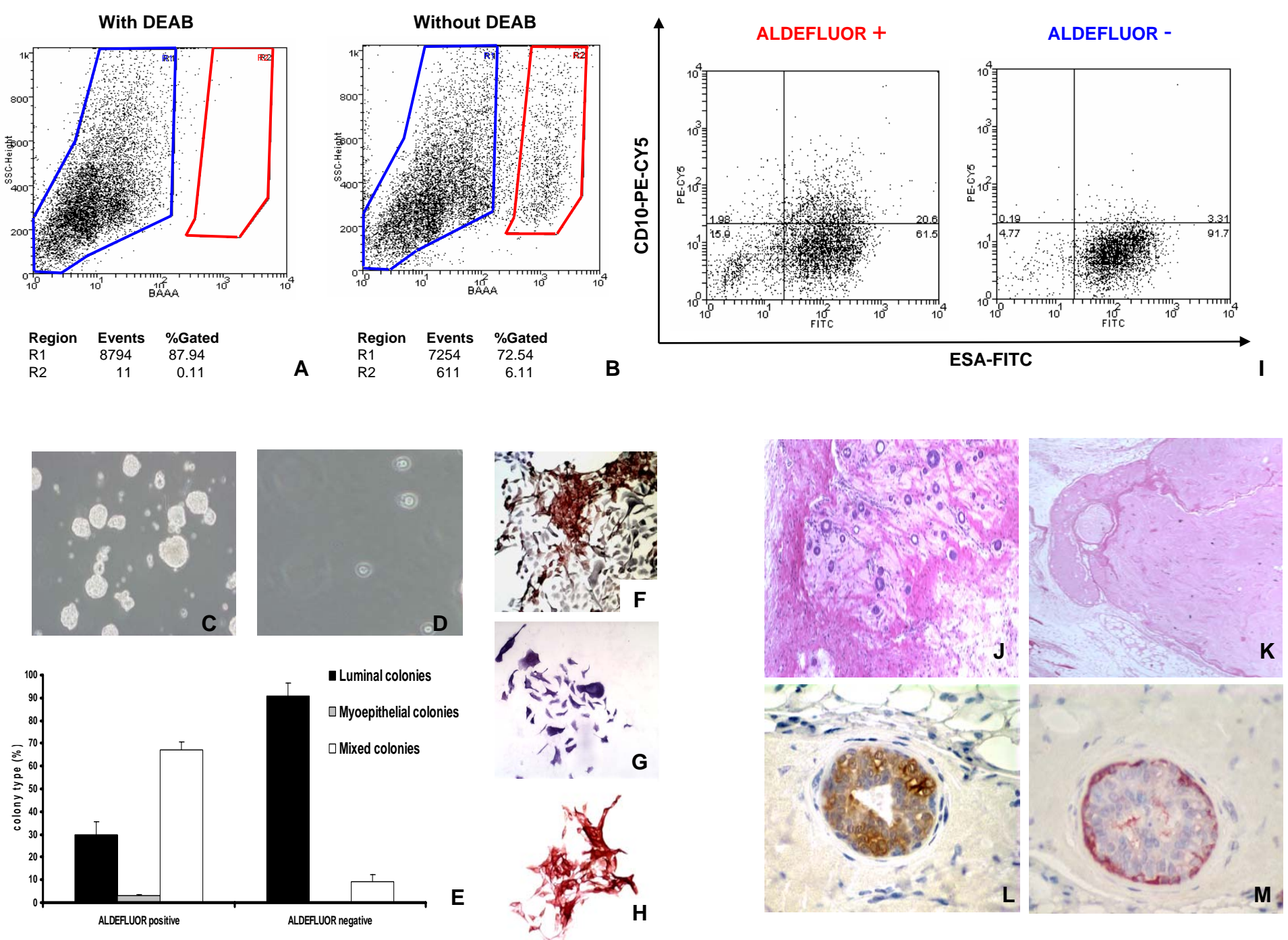
**Figure 3. The ALDEFLUOR positive cell population displays properties of cancer stem cells in NOD/SCID mice.** **A-B.** Representative flow cytometry analysis of ALDH activity in cells derived from a human breast tumor, orthotopically xenotransplanted in NOD/scid mice. Cells incubated with ALDEFLUOR substrate (BAAA) and the specific inhibitor of ALDH, DEAB, were used to establish the baseline fluorescence of these cells (R1) and to define the ALDEFLUOR-positive region (R2) (**A**). Incubation of cells with ALDEFLUOR substrate in the absence of DEAB induced a shift in BAAA fluorescence defining the ALDEFLUOR-positive population (**B**). **C-G** Only the ALDEFLUOR-positive population was tumorigenic. **C.** The ALDEFLUOR-positive population was capable of regenerating the phenotypic heterogeneity of the initial tumor after a passage in NOD/scid mice. **D.** Tumor progression curves were plotted for the numbers of cells injected (50,000 cells; 5,000 cells; 500 cells) and for each population

(ALDEFLUOR-positive, ALDEFLUOR-negative, unsorted). Tumor growth kinetics correlated with the latency and size of tumor formation and the number of ALDEFLUOR-positive cells. **E.** Representative tumor grown in NOD/scid mouse at the ALDEFLUOR-positive cells' injection site (5,000 cells injected). No tumor was detected at the ALDEFLUOR-negative cells' injection site (5,000 cells injected). **F-G.** H & E staining of ALDEFLUOR-positive cells' injection site revealing presence of tumor cells (**F**). The ALDEFLUOR-negative cells' injection site contained only residual Matrigel, apoptotic cells and mouse tissue (**G**). All the data presented in this figure were generated by analysis of the MC1 tumor. Similar results were obtained for three other tumors, generated from different patients (UM1, UM2, and UM3) tested (supplementary figure 2).

**Figure 4. Expression of ALDH1 in breast carcinomas, as shown by immunohistochemistry on tissue microarrays (TMA), using two independent tumor sets. A-D.** Example of ALDH1 staining in breast cancer. Only two of the 477 tumors analyzed were fully positive for ALDH1 (**A**). Representative examples of breast tumor cores positive for ALDH1 with 5-10% ALDH1-positive cells detected (**B-C**). Example of a tumor core with no detectable ALDH1 staining (**D**). **E-F.** Kaplan-Meier plot of patient overall survival: Survival differed significantly according to ALDH1 expression. Patients with tumors positive for ALDH1 staining (green curve) had a poor prognosis compared to patients with tumors negative for ALDH1 staining (blue curve). Similar results were observed in the U.M. set composed of 136 patients ( $p=0.0459$ ) (**E**) and I.P.C. set composed of 341 patients ( $p=0.000675$ ) (**F**). **G.** Cox multivariate analysis of overall survival for patients from I.P.C. set. When compared with known prognostic factors, ALDH1 status was an independent factor of prognosis, as was Ki-67 status, tumor size, SBR grade.

## Abstract

Application of stem cell biology to breast cancer research has been limited by the lack of simple assays for identification and isolation of normal and malignant stem cells. We show that stem/progenitor cells in normal breast epithelium and breast tumors have increased aldehyde dehydrogenase activity. Furthermore, immunostaining using ALDH1 antibody identifies normal and malignant stem/progenitor cells in situ. In a series of 577 breast carcinomas on tissue microarrays, expression of ALDH1 was an independent predictor of poor prognosis. These findings provide support for the “cancer stem cell hypothesis” and offer an important new tool for the study of normal and malignant breast stem cells. Moreover, since ALDH1 immunodetection provides a simple method to identify cancer stem and progenitor cells in situ it should facilitate the clinical application of stem cell concept.



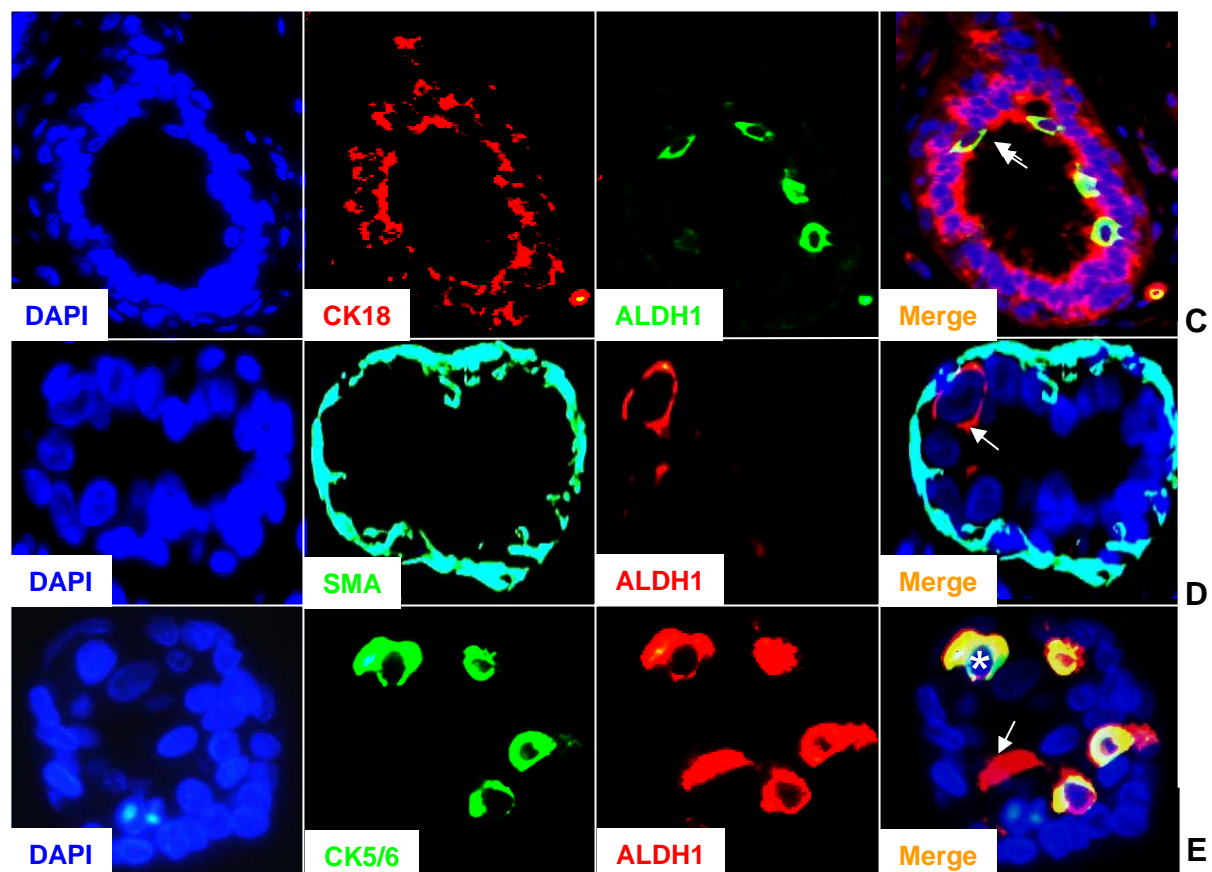
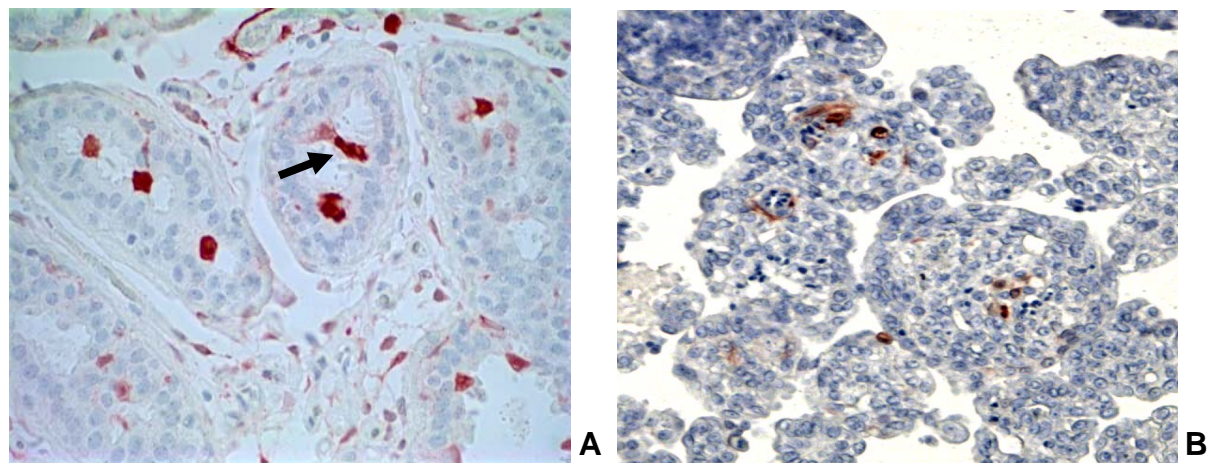


Figure 2

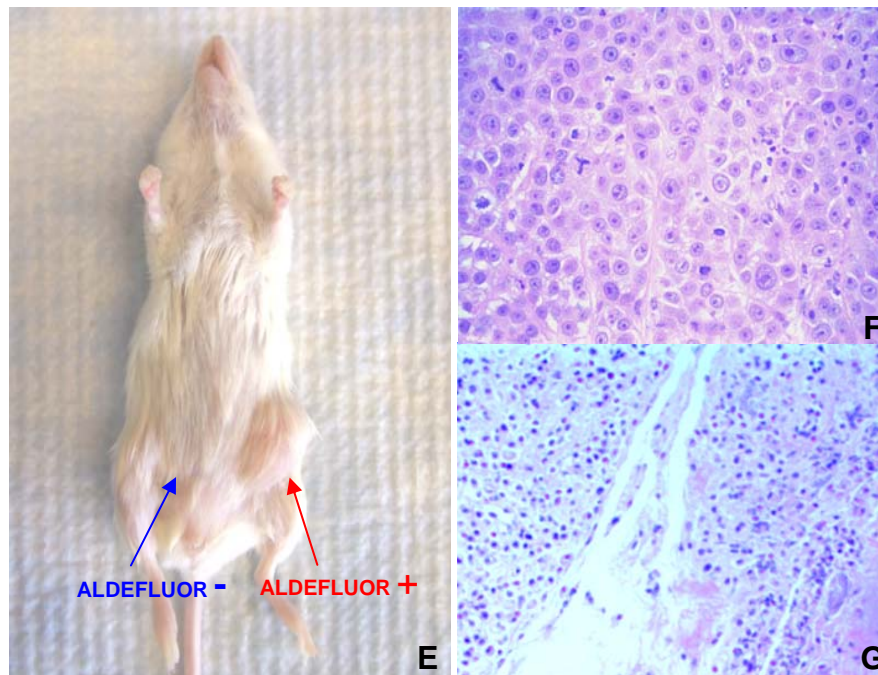
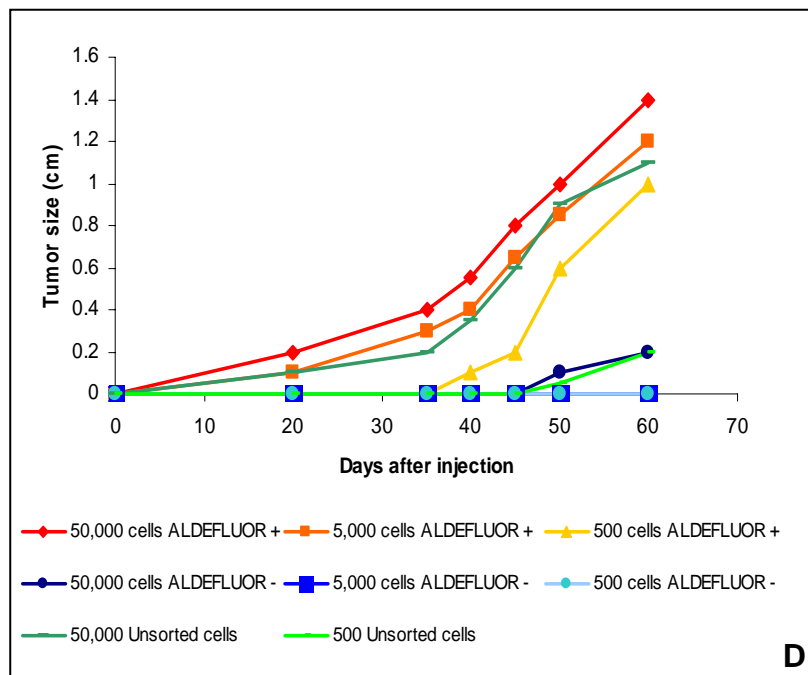
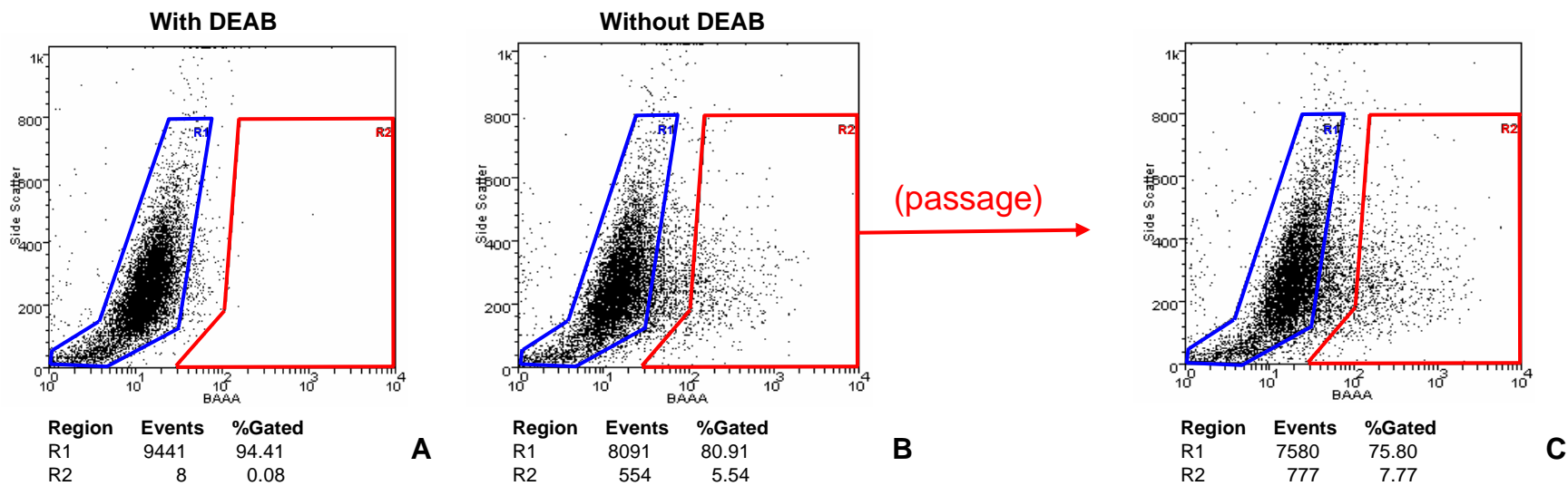
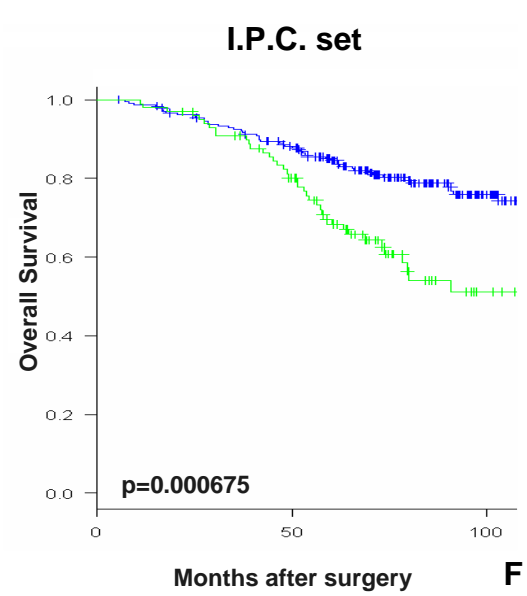
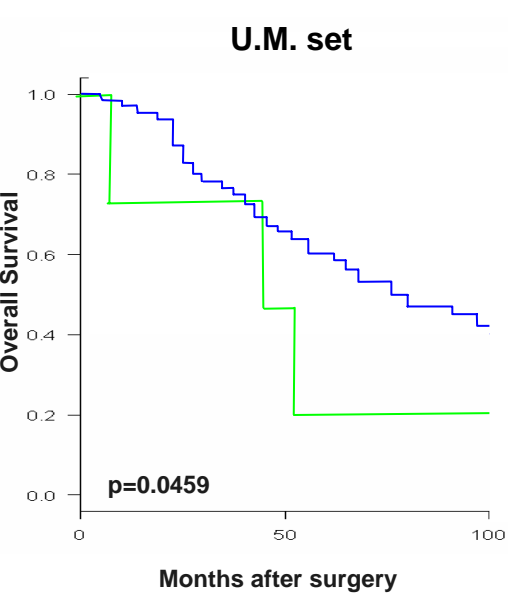
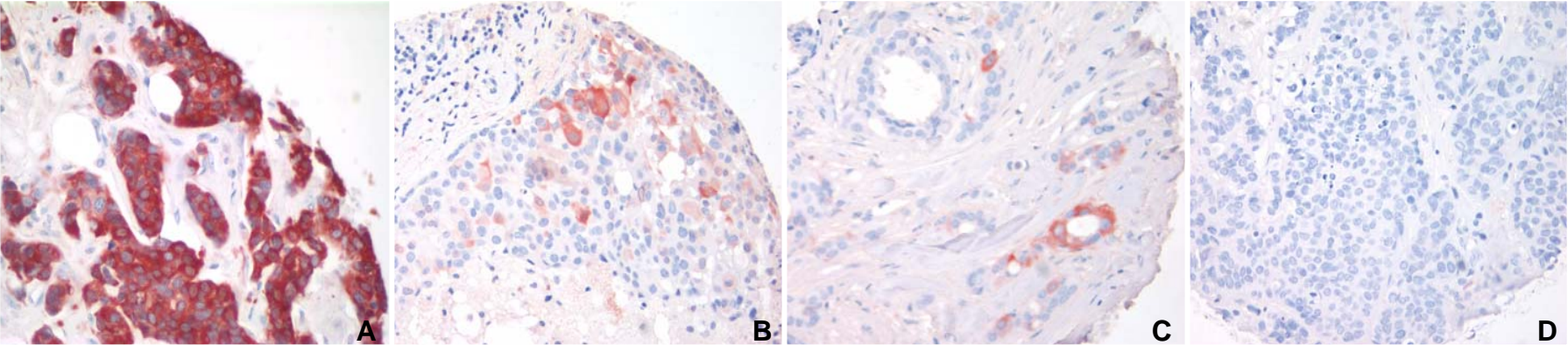


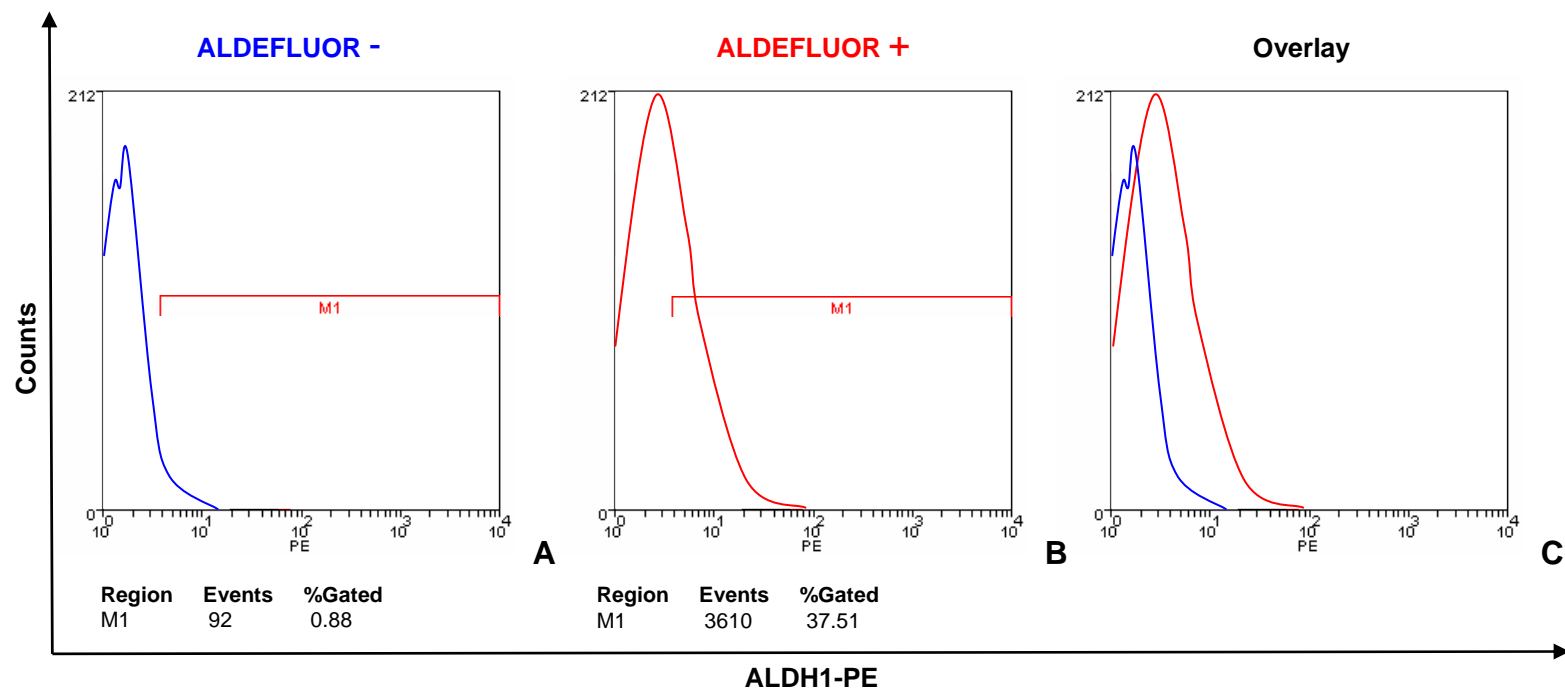
Figure 3



Variable	Hazard ratio (95% CI)	p-value
Ki-67 status		
Negative	1	0.0029
Positive	2.47 (1.36-4.47)	
Tumor size (mm)		
≤20	1	0.0093
>20	2.21 (1.22-4.02)	
SBR Grade		
I and II	1	0.025
III	1.83 (1.08-3.1)	
ALDH1 status		
Negative	1	0.028
Positive	1.76 (1.06-2.9)	

**G**

Figure 4



# UM1

# UM2

# UM3

With DEAB

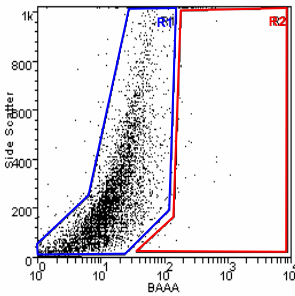
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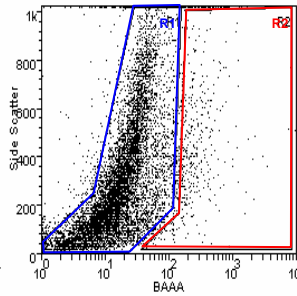
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With DEAB

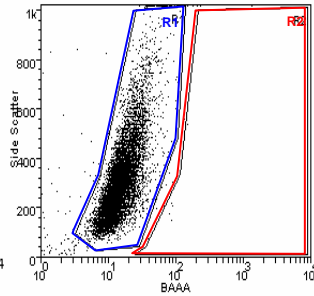
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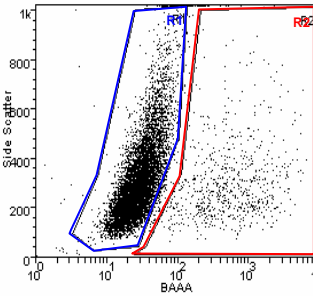
Region	Events	%Gated
R1	8976	89.76
R2	14	0.14



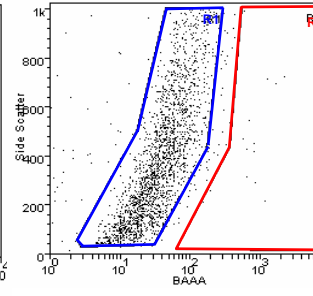
Region	Events	%Gated
R1	7894	78.94
R2	327	3.27



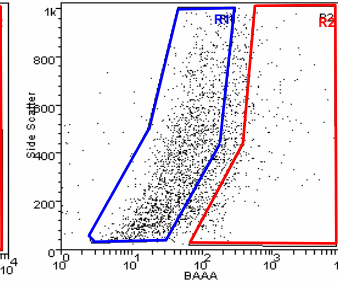
Region	Events	%Gated
R1	8245	82.45
R2	9	0.09



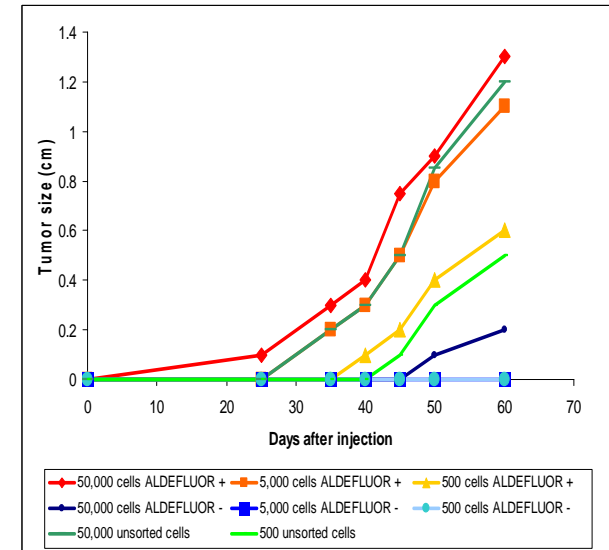
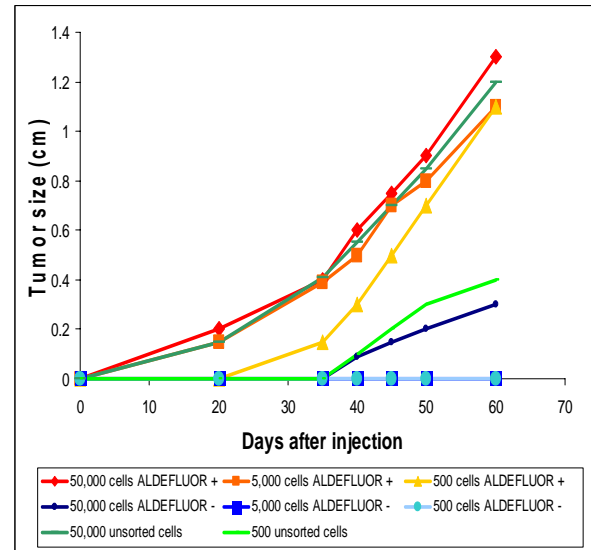
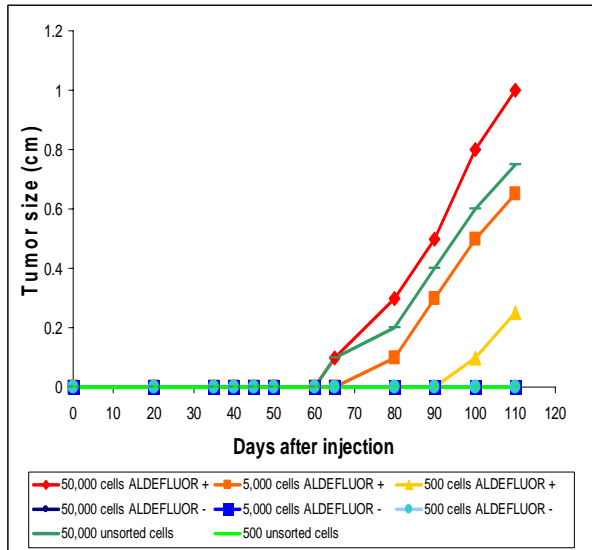
Region	Events	%Gated
R1	7245	72.45
R2	896	8.96



Region	Events	%Gated
R1	8921	89.21
R2	13	0.13



Region	Events	%Gated
R1	6553	65.53
R2	938	9.38



**Supplementary Figure 1. Overlap between the normal mammary epithelial cell populations with ALDH activity detected by the ALDEFLUOR assay and the cell population with cytoplasmic ALDH1 protein detected by immunostaining.** ALDEFLUOR-negative and ALDEFLUOR-positive cells from normal breast epithelium were separated by FACS using the ALDEFLUOR assay. Cells were then fixed in RNA later, immunostained with an ALDH1 antibody and analyzed by flow cytometry. **(A-C)**. ALDEFLUOR-negative cells did not have levels of ALDH1 protein detectable by immunostaining (M1=0.88%) **(A)** whereas ALDEFLUOR-positive cells contained all the cell population detected by ALDH1 antibody **(B)**. Overlay presenting a direct comparison between ALDH1 immunostaining of ALDEFLUOR-negative and ALDEFLUOR-positive cells **(C)**.

**Supplementary Figure 2. The ALDEFLUOR positive cell population displays properties of cancer stem cells in NOD/SCID mice.** Representative flow cytometry analysis of ALDH activity in cells derived from a human breast tumor, orthotopically xenotransplanted in NOD/SCID mice (UM1, left panel; UM2, central panel; UM3 right panel). Cells incubated with ALDEFLUOR substrate (BAAA) and the specific inhibitor of ALDH, DEAB, were used to establish the baseline fluorescence of these cells (R1) and to define the ALDEFLUOR-positive region (R2). Incubation of cells with ALDEFLUOR substrate in the absence of DEAB induced a shift in BAAA fluorescence defining the ALDEFLUOR population. Tumor progression curves were plotted for the numbers of cells injected in NOD/SCID mice (50,000 cells; 5,000 cells; 500 cells) and for each population (ALDEFLUOR-positive, ALDEFLUOR-negative, unsorted).

**Supplementary Table 1. Outgrowth potential in the humanized fat pad of NOD/SCID mice**

	Outgrowth/injections			
	5x104	2.5x104	5x103	500
ALDEFLUOR-positive	---	4/4	3/4	0/4
ALDEFLUOR-negative	0/4	---	0/3	0/2
Unsorted	---	5/5	3/5	0/2

## **Selective targeting of cancer stem cells: A new concept in cancer therapeutics**

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## **Abstract**

Although the concept of “cancer stem cell” had been proposed more than a century ago, it has attracted a great deal of attention recently due to advances in stem cell biology leading to the identification of these cells in a wide variety of human cancers. Furthermore, there is accumulating evidence that the resistance of cancer stem cells to many conventional therapies may account for the inability of these therapies to cure most metastatic cancers. The recent identification of stem cell markers and advances in stem cell biology has facilitated research in multiple aspects of cancer stem cell behavior. Stem cell subcomponents have now been identified in a number of human malignancies including haematologic malignancies and tumors of the breast, prostate, brain, pancreas, head and neck and colon. Furthermore, pathways that regulate self-renewal and cell fate in these systems is beginning to be elucidated. In addition to pathways known to regulate self-renewal of normal stem cell such as Wnt, Notch and Hedgehog, tumor suppressor genes such as PTEN and p53 have also been implicated in the regulation of cancer stem cell self-renewal. In cancer stem cells, these pathways are believed to be deregulated leading to uncontrolled self-renewal of cancer stem cells which generate tumors that are resistant to conventional therapies. Current cancer therapeutics based on tumor regression may target and kill differentiated tumor cells which compose the bulk of the tumor while sparing the rare cancer stem cell population. The cancer stem cell model suggests that the design of new cancer therapeutics may require the targeting and elimination of cancer stem cells. Therefore, it is imperative to design new strategies based upon a better understanding of the signaling pathways that control aspects of self-renewal and survival in cancer stem cells in order to identify novel therapeutic targets in these cells.

## **Introduction**

“Decades of cancer research may need to be re-evaluated, because standard tumor-targeting therapies may be off the mark, mounting research suggests.” Quote from ABC news, November 2006, voicing the concerns over the failure of current cancer therapies to cure the most common human cancers and posing the question of

whether we are targeting the right cells in human cancers. Conventional therapies have largely been designed to target bulk and cycling populations in tumors. Evidence is accumulating from number of human malignancies that most, if not all, malignancies possess a subcomponent of cancer cells that have stem cell properties which have been “cancer stem cells” (CSC). These properties include self-renewal that drives tumorigenesis and differentiation that generates the bulk of tumor cells. The deregulation of stem cell self-renewal pathways through accumulation of both genetic and epigenetic changes may be essential for the malignant transformation of CSCs (1-3). During normal development, signals from the surrounding niche regulate stem cell self-renewal. The altered reorganization of these niches may result in aberrant signals that lead to deregulation of stem cell self-renewal (4). This concept is supported by a recent finding which demonstrated an increase in self-renewing CSC population by increasing the vasculature in the brain tumor microenvironment (5). On the contrary, the depletion of blood vessels from xenografts ablated self-renewing tumor stem cells and inhibited tumor growth (5). Thus the aberrant regulation of stem cell self-renewal due to both extrinsic and intrinsic signals may generate the malignant phenotype. Although the processes that control self-renewal are complex and are only beginning to be understood, the concept of CSCs has fundamental implications for understanding the tumor biology as well as developing new strategies to combat cancer. This review will discuss the evidence for existence of cancer stem cells in a variety of human malignancies and implications of the cancer stem cell model for the development of new cancer therapeutics. A model which illustrates the rationale for the development of stem cell targeted therapies summarized in Figure 2.

### **Isolation of normal adult and cancer stem cells**

Stem cells are defined by their two distinct properties; (a) self-renewal characterized by the ability to go through numerous cycles of cell division while maintaining an undifferentiated state and (b) multipotency or the ability to generate progeny of distinct cell types (6, 7). Tissue specific (adult) stem cells are distinguished from embryonic stem cells in that their ability to differentiate is largely restricted to cell types within a particular organ. Although transdifferentiation (plasticity) of adult stem cells into different tissues such as the brain or blood cells into mature cells of different tissues

has been reported, this apparent plasticity is often the result of a rare fusion of stem/progenitor cells of different origin (8-10). HSCs identified by cell surface marker expression from human and mouse (11-13) were able to reconstitute the haematopoietic system. The expansion and regeneration of mammary epithelium during puberty and pregnancy in reproductive cycles suggests the existence of stem/progenitor cells. Kordon et al, first described the repopulation of mouse mammary gland through serial transplantation of retrovirally tagged epithelial fragments (14) demonstrating the clonal nature of this repopulation. More recently, generation of a functional mammary gland from a single stem cell has also been described (15). In normal tissues, homeostasis is tightly regulated to ensure the generation of mature cells throughout life without depletion of stem cell pools (16). Each tissue is comprised of a cellular hierarchy including stem cells able to generate all progeny, committed progenitors and terminally differentiated cells. While stem cell self-renewal is necessary for tissue repair and regeneration, it also carries the risk of genetic alteration in stem cells due to the error-prone nature of DNA replication. Thus the pathways that control stem cell self-renewal and the microenvironment in which the stem cells reside both may play a role in carcinogenesis (17). Deregulation of self-renewal and subsequent loss of homeostasis may result in malignant transformation of human tissues and this forms the basis of cancer stem cell hypothesis. The concept of cancer stem cell was first proposed by Virchow and Cohnheim almost 150 years ago based on the similarities between fetal development and certain types of tumors such as teratocarcinomas (1). John Dick and his colleagues were the first to isolate such cells from acute myeloid leukaemias where a small subset of CD34<sup>+</sup>CD38<sup>-</sup> cells that comprised <1 in 10,000 cells could transfer human leukemia into NOD-SCID mice whereas the remaining population that did not bear this phenotype failed to do so (18). Furthermore, this group demonstrated the heterogeneity of leukemia CSCs with hierarchical self-renewal potential reminiscent of their normal counterparts (19). Similar techniques have been used to demonstrate a similar cellular hierarchy in solid tumors including breast, prostate, brain, pancreas and colon (20-25). Implantation of small subsets of cells from these solid tumors revealed that only the cells with stem cell characteristics were able to form tumors suggesting the existence of CSCs in these tumors. For example, in

collaboration with Michael Carke, we demonstrated that human breast cancers contain a stem cell population characterized by the expression of cell surface markers  $CD44^+CD24^{low}Lin^-$ . As few as 200 of these cells, comprising 1 to 10% of total cell population were capable of forming tumors when implanted in NOD-SCID mice. In contrast, 20,000 cells that did not express these markers were unable to form tumors (22). Consistent with the cancer stem cell model, the stem cells were able to generate tumors that recapitulated the phenotypic heterogeneity of initial tumor. We and others confirmed that breast CSCs are not only tumorigenic but also formed mammospheres *in vitro*, a property that we described previously for normal mammary stem/progenitor cells (26, 27). Interestingly, these cells also expressed a stem cell marker, Oct-4 lending additional support to CSC hypothesis (26). Identification and subsequent use of a cell surface antigen, CD133, a five transmembrane glycoprotein (28) enabled Uchida et al, to isolate human central nervous system stem cells characterized by  $CD133^+CD34^-CD45^-$  expression (29). Through serial dilution, this group demonstrated that a single  $CD133^+CD34^-CD45^-$  cell was able to form a neurosphere in *in vitro* culture (29). In addition to the identification of normal neuronal stem cells, the existence of CSCs in brain tumors have also been reported (30-32). Through cell sorting for  $CD133^+$ , a functional cellular hierarchy in brain tumor cell population was demonstrated (33). Furthermore,  $CD133^+$  human brain tumor cells but not  $CD133^-$  were able to form tumors in NOD-SCID mouse brains and neurospheres in *in vitro* cultures (21). Xin et al, demonstrated that prostate regenerating cells are enriched in stem cell antigen-1 (Sca-1) expression (34). Further evidence for the existence of CSC population in human prostate tumors has been reported (35). Richardson et al, has identified a stem cell population in normal human prostate characterized by  $CD133^+$  expression and in human prostate tumors characterized as  $CD44^+/\alpha_2\beta_1^{hi}/CD133^-$  (35). As few as 500 cells with this phenotype that constituted 0.1% of total tumor cells formed tumors in NOD/SCID mice, whereas  $5 \times 10^5$   $CD44^-$  cells failed to form tumors (35). The existence of stem cells in normal lung and lung cancer has also been shown by the isolation of cells that exhibited self-renewal and multipotency (36). Most recently, the identification and characterization of cancer stem cell populations in colon tumors has been reported (23, 24). Ricci-Vitiani et al, and

O'Brien et al, isolated CD133<sup>+</sup> and CD133<sup>-</sup> cells from number of human colon cancers and injected either subcutaneously or under the renal capsule of NOD-SCID mice. Both groups independently demonstrated that CD133<sup>+</sup> cells were not only capable of tumor formation but that they also re-established the original tumor heterogeneity (23, 24). This is consistent with the cancer stem cell hypothesis that suggest that tumors are generated and maintained by a small subset of undifferentiated cells that are able to self-renew and differentiate to generate cells which constitute the bulk of tumor (37). Although cancer stem cells and their differentiated progeny carry the same oncogenic mutations, these more differentiated cells are non-tumorigenic due to their inability to self-renew (17).

Although progress has been made in identifying cancer stem cells from variety of human malignancies, the pathways which drive transformation of these cells are poorly understood. Since transformation appears to be caused by mutations that dysregulate normal stem cell self-renewal, it is critical to understand the pathways that regulate this process (38). Increased self renewal and decreased differentiation of stem cells would be expected to lead to an increase in stem cell pools. This has also been termed "maturation arrest" or "blocked ontogeny" as oppose to dedifferentiation of mature cells (39). As early as 1950's, J. Furth proposed the acquired inability of immature leukocytes to respond to forces normally regulating their proliferation and maturation (40). It is now widely accepted that the idea of "maturation arrest" through arrested differentiation of tissue-based stem cells or their immediate progeny is closely linked to the development of human malignancies (41). Therefore a great deal of research to better understand the self-renewal and differentiation pathways of normal and cancer stem cells is underway.

### **Self-renewal pathways that are dysregulated in cancer stem cells**

Stem cells in their microenvironment are maintained through infrequent and mainly asymmetric divisions by which a stem cell gives rise to two daughter cells with distinct fates: one is the exact copy of parent, another is programmed to differentiate. Stem cells in self-renewing mammalian epithelium are believed to exert an axis of polarity. Asymmetric cell division takes place perpendicular to this axis, regenerating the stem cells as well as generating a committed daughter cell (42). These processes are

relatively well studied in *Drosophila* and suggest interesting links between stem cell self-renewal and transformation (43-45). Disruption of asymmetric cell division in *D. melanogaster* impaired the polarity and induced neoplastic growth in epithelia and neurons (46, 47). Based on studies in flies and initial observations in mammalian stem cells, alteration of self-renewal pathways appears to be an important mechanism underlying the malignant transformation resulting in the generation of CSCs.

### **Pathways involved in stem cell self-renewal**

**Hedgehog signaling:** One of the signaling pathways implicated in embryonic development is Hedgehog (Hh) first identified in drosophila screen for genes that were required for patterning of the early embryo (48). Subsequent identification of three Hh members including Sonic (Shh), Desert (Dhh) and Indian (Ihh) in mammals led to the demonstration of its role in the development of human malignancies (49, 50).

Dahmane et al. demonstrated a layer-specific expression of Shh in the perinatal mouse neocortex and tectum while that the expression of Gli genes were limited to the proliferative zones (51). Thus Shh serves as a mitogen for neocortical and tectal precursors which mediates cellular proliferation in the dorsal brain (51). Moreover, Hh-Gli pathway regulates homeostasis in embryonic and adult mouse neocortical stem cells by co-operation with EGF signaling (52). Palma et al. reported similar findings that Hh-Gli pathways is required for proliferation of mouse forebrain's subventricular zone (SVZ) stem cell niche and for the production of new olfactory interneurons in vivo (53, 54). Abrogation of Shh signaling resulted in dramatic reduction of number of neural progenitors in both the postnatal subventricular zone and hippocampus (55). Deregulation of the Hedgehog (Hh) pathway has been reported for number of human malignancies including basal cell carcinoma (BCC), medulloblastoma, glioma, colon, prostate, small cell lung cancer, pancreatic and breast cancers (50, 56-61). Although rare, mutations of Shh were found in BCC, medulloblastomas and breast carcinomas (58). However, another study found no missense mutations of Ptch and Shh in 84 primary breast carcinomas (62). Hh ligands bind to Patched1 (Ptch1) and Patched2 (Ptch2) transmembrane receptors (63, 64). The ligation of Hh with Ptch receptors relieves the inhibitory effect of Ptch on another transmembrane protein, Smoothed (Smo) and subsequently induces the activation of Gli transcription factors (65, 66). We

have recently demonstrated that Hh signaling components, *Ptch1*, *Gli1* and *Gli2* are highly expressed in normal human mammary epithelial stem/progenitor cells while downregulated in differentiated cells (67). Activation of hedgehog signaling increases mammosphere-initiating cell number and mammosphere size, conversely inhibition of the pathway results in a reduction of these effects. These effects are mediated by the polycomb gene *Bmi-1*. Furthermore, overexpression of *Gli2* in mammosphere-initiating cells results in the formation of ductal hyperplasia, and modulation of *Bmi-1* expression in mammosphere-initiating cells alters mammary development in a humanized immunodeficient mouse model (67). *Gli1* originally was identified as a gene which was amplified in human glioma (68). Ectopic expression of *Gli1* or *Gli2* in the skin of *xenopus* or mice results in tumor formation (69, 70). Hh signaling components were undetectable in normal human ductal epithelium but are strongly expressed in precursor cells and invasive lesions and recently an abnormal Hh expression has also been reported in pancreatic cancer stem cells (25, 71).

**Notch signaling:** Notch signaling was first discovered in flies due to loss of function which resulted in notches at the wing margin. In flies, the *Notch* gene encodes a 300 kD transmembrane receptor with 36 tandem epidermal growth factor receptor-like repeats and three cysteine-rich Notch/LIN-12 repeats in its extracellular domain (72). Notch proteins, Notch1-4 encoded by four homologous genes and two Notch ligands, Delta and Jagged, have been identified in vertebrates (73, 74). Notch is known to promote the survival and proliferation of neural stem cells through inhibition of their differentiation (75, 76). Notch also plays role in brain development, a transient administration of Notch ligands to the brain of adult rats increases the number of newly generated precursor cells and improves motor skills after ischemic injury (77). Binding of ligand to a notch receptor initiates three proteolytic cleavages, two cleavages take place at the extracellular domain of Notch followed by third cleavage by a  $\gamma$ -secretase complex in the plasma membrane that releases the intracellular domain of the receptor into cytoplasm (78). This intracellular domain of Notch then translocates into nucleus to transcribe a number of target genes. Inhibitors of the  $\gamma$ -secretase complex deplete stem cells and slow the growth of Notch-dependent tumors such as medulloblastoma and T-cell leukemia (79-82). We have previously

demonstrated that induction of Notch signaling promotes self-renewal of mammary stem cells via increasing cellular proliferation of stem and early progenitor cells. We observed a 10-fold increase in secondary mammosphere formation after treatment with Notch-activating DSL peptide. Activation of this pathway also increased branching morphogenesis in three-dimensional matrigel cultures. These effects were completely blocked by a Notch blocking antibody or  $\gamma$ -secretase inhibitor suggesting a specific requirement of Notch in these signaling events (83). The vertebrate Notch4 gene has been shown to be involved in normal mammary development (84). In vitro, overexpression of a constitutively active form of Notch4 inhibits differentiation of normal breast epithelial cells (85). In vivo, transgenic mice expressing a constitutively active form of Notch4 fail to develop normal mammary glands and subsequently develop mammary tumors (86). In contrast, Notch1 may also function as tumor suppressor in a tissue specific fashion. Nicolas et al. demonstrated that Notch1 inactivation results in increased Gli2 expression and subsequently development of basal-cell carcinoma-like tumors (87).

**Wnt pathway:** The Wnt (wingless gene) was originally identified as a segment polarity gene in *Drosophila melanogaster* that functions during embryogenesis (48). The canonical Wnt pathway regulates a number of events in cells by binding to cell-surface receptors of the Frizzled family, resulting in activation of Dishevelled family of proteins and ultimately nuclear translocation of  $\beta$ -catenin (Figure 1). Dishevelled (DSH) is a key component of a membrane-associated Wnt receptor complex which inhibits the axin/GSK-3/APC protein complex. DICKKOPF-1 (DKK-1) encodes a secreted Wnt antagonist that binds to LRP5/6 and induces its endocytosis, leading to inhibition of the canonical pathway (88). The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the  $\beta$ -catenin (Figure 1). However inhibition of the  $\beta$ -catenin destruction complex leads to stabilization and nuclear translocation of  $\beta$ -catenin where it interacts with TCF/LEF family of transcription factors to promote specific gene expression. Growing number of Wnt/ $\beta$ -catenin pathway target genes have been identified (<http://www.stanford.edu/~rnusse/pathways/targets.html>). Among those *cyclin D*, *c-myc*, *metalloproteinases*, *c-met*, *VEGF* and *Jagged1* are implicated in tumorigenesis. The Wnt pathway is absolutely required for embryonic development.

Mice deficient in any Wnt pathway components such as Wnt3, LRP5/6 or  $\beta$ -catenin fail to develop a primitive streak and lack mesoderm (89, 90). Li et al. demonstrated the expansion of an epithelial cell population expressing progenitor cell markers, keratin 6 and Sca-1 in MMTV-Wnt transgenic mice (91). However, this phenotype was lacking in MMTV transgenic mice expressing Neu, H-Ras or polyoma middle T antigen (91). Furthermore, loss of *Lrp5* significantly reduced early proliferation of progenitor cells and subsequent formation of mammary tumors in *MMTV-Wnt1* transgenic mice indicating a role for *Lrp5* in Wnt signaling (92). Intestinal stem cells have been identified by using a 5-bromodeoxyuridine (BrdU)-retaining assay (93). These cells are located at the bottom of each crypt (93). Nuclear  $\beta$ -catenin accumulates at the bottom of normal adult crypts in small intestine and colon where the stem/progenitor cells reside (94, 95). Moreover, transgenic expression of the Wnt specific inhibitor Dkk-1, in the intestine of adult mice reduces epithelial proliferation with subsequent loss of crypts as well as ablation of secretory cell lineages (96). This suggest a role for the Wnt pathway in the maintenance of intestinal stem cells. Bone morphogenetic protein, BMP signaling also plays key role in gastrointestinal development and maintenance of adult tissue homeostasis. He et al. demonstrated that the conditional deletion of *Bmpr1a* in mice results in expansion of stem/progenitor cells and development of intestinal polyposis resembling the human juvenile polyposis syndrome caused by germline nonsense mutations of *Bmpr1* (97). Studies to understand the pathways that regulate haematopoietic stem cell self-renewal revealed a requirement for Wnt signaling (98). Overexpression of activated beta-catenin not only expands the pool of HSCs in long term cultures but also activates the LEF-1/TCF reporter suggesting that HSCs respond to Wnt signaling *in vivo* (98). Altogether these results strongly suggest that the Wnt pathway plays a key role in self-renewal of adult stem cells and that deregulation of the pathway is involved in carcinogenesis.

**PTEN pathway:** Since the discovery of the tumor suppressor gene, phosphatase and tension homolog on chromosome ten (PTEN), (99, 100) a number of studies placed the protein in the center of complex signaling networks. Mutations or allelic loss of PTEN has been found in a large number of human malignancies including brain, breast and prostate (100-103). In addition, germ line mutations of PTEN cause rare

inherited diseases including Cowden syndrome which is associated with development of malignant tumors (104). PTEN acts as a lipid phosphatase to dephosphorylate phosphatidylinositol-triphosphate (PIP3) that antagonizes the PI3-K/Akt pathway (Figure 1). Inhibition or deletion of PTEN results in increased activation of the PI3-K/Akt pathway which in turn phosphorylates a number of substrate proteins. In addition to its role in cell-cycle regulation, Akt also phosphorylates and inactivates GSK3- $\beta$  which is involved in the regulation of Wnt signaling (105). Akt has also been shown to directly phosphorylate  $\beta$ -catenin at Ser 552 which promotes its nuclear transport. Thus activation of Akt promotes the Wnt signaling resulting in nuclear accumulation of  $\beta$ -catenin as illustrated in Figure 1 (106). Furthermore increased Akt activation in breast cancer patients predicts poor prognosis (107). Deletion or reduced PTEN expression in wide range of human tumors predicts resistance to conventional therapies and a relapse following initial regression (108, 109). Shamon et al, have reported a strong correlation between downregulation of PTEN expression and failure to respond tamoxifen treatment in 100 ER $\alpha$ -positive tumors treated with tamoxifen (108). In prostate tumors, loss of PTEN expression also predicts progression towards invasive and metastatic disease (110). These results further support the concept of CSC since they suggest that current cancer therapies do not target CSCs, only differentiated cells will be eliminated and the residual tumors containing CSCs will reconstitute the tumor. Deletion of PTEN in murine model of prostate cancer resulted in expansion of the prostate stem/progenitor cell population and initiated prostate tumors resembling those in humans (111). In the haematopoietic system, Yilmaz et al. and Zhang et al. have recently reported that conditional deletion of the PTEN tumor suppressor gene resulted in excessive proliferation of HSCs and their subsequent depletion in bone marrow (112, 113). Thus PTEN deficiency results in myeloproliferative disorders and eventually leukemia (112, 113). The most recent study by He et al, using conditional deletion of PTEN demonstrated the expansion of intestinal stem cells and formation of intestinal polyposis in a mouse model (114). This further indicates that as a tumor suppressor, PTEN might play a key role in maintaining the homeostasis in variety of tissues through regulating stem cell self-renewal.

**p53 pathway:** The tumor suppressor p53, its downstream target p21 and its regulator p19<sup>ARF</sup> have all been implicated in the regulation of stem cell self-renewal (115-118). The majority of human malignancies display either p53 mutations or dysregulation of the p53 pathway (119, 120). In response to stress signals, such as UV irradiation and DNA damaging agents, p53 becomes activated and promotes cell-cycle arrest or apoptosis depending on the signal. In embryonic stem cells (ESC), however, the p53 cascade appears to play a different role. Despite abundant accumulation of p53 in response to DNA damage, ESCs from wild type mice did not activate a p53-dependent stress responses (121). Lin et al, suggested that activated p53 binds to the promoter of *Nanog*, a gene required for ESC self-renewal (122, 123), and suppresses *Nanog* expression after DNA damage. The rapid downregulation of *Nanog* expression during differentiation correlates with the induction of p53 transcriptional activity and Ser 315 phosphorylation (124). Meletis et al, recently reported that p53 suppresses self-renewal of adult neural stem cells as demonstrated by increased neural stem cell proliferation *in vivo* and increased neurosphere formation of cells *in vitro* from p53 null mice brain as compare to that of wild type mice (125). One of the p53-transcriptional target genes, p21 has been implicated in maintenance of HSC quiescence. In p21 null mice, baseline HSC self-renewal is increased. However, exposing animals to cell cycle specific myelotoxic injury resulted in premature death due to rapid depletion of HSCs (118). It is believed that p21 functions as a molecular switch regulating the cell cycle entry of stem cells. In its absence increased cell cycling causes extensive cellular proliferation leading exhaustion of HSCs. The mammalian INK4a/ARF locus encodes two tumor suppressor proteins: the cyclin-dependent kinase inhibitor p16<sup>INK4a</sup> and p19<sup>ARF</sup>, a potent regulator of p53 stability. Further examination of the role of these two genes revealed that expression of p16<sup>INK4a</sup> and p19<sup>ARF</sup> resulted in proliferative arrest and p53-dependent cell death (126). In Bmi-1 null mice, a relationship between stem cell self-renewal and cellular ageing may also involve p16<sup>INK4a</sup> (115). HSCs in older mice have decreased self-renewal and increased cell death in response to stress (127, 128). Janzen et al, have tested the levels of p16<sup>INK4a</sup> in HSCs characterized by Lin<sup>-</sup>Kit<sup>+</sup>Sca1<sup>+</sup>CD34<sup>low</sup>FLK-2<sup>low</sup> from two strains of young and old mice and demonstrated that p16<sup>INK4a</sup> mRNA was not detectable in young mice, whereas in

old animals increased p16<sup>INK4a</sup> mRNA levels were observed (129). Consistent with these findings, Molofsky et al, reported that ageing p16<sup>INK4a</sup> wild type mice demonstrated significantly more decline in subventricular zone proliferation, olfactory bulb regeneration and self renewal when compared to p16<sup>INK4a</sup> deficient mice (130). Animal models of tumor recurrence recently have provided some clues as to pathways that might be involved. Doxycycline-inducible *Wnt1* transgenic mouse model (MTB/TWNT) of mammary adenocarcinomas depends on continued Wnt signaling and downregulation of Wnt pathway results in rapid disappearance of primary mammary tumors as well as pulmonary metastasis (131). However, a significant fraction of tumors progress to a Wnt independent state. Studies to further investigate molecular pathways involved in the re-growth of residual tumors showed that the majority of regressed tumors exhibited complete or partial LOH at the *p53* locus implying a selective loss of the wild type *p53* allele. Furthermore, almost all tumors with MTB/TWNT/*p53*<sup>+/+</sup> regressed to non-palpable state following doxycycline withdrawal, whereas 40% of tumors arising from MTB/TWNT/*p53*<sup>+/-</sup> mice failed to regress suggesting a specific role for *p53* (131). Most recently two different studies have demonstrated that in *p53* deficient tumors the restoration of *p53* results in tumor regression or arrest of tumor growth (132, 133).

**Therapeutic targeting of CSCs:** The lack of substantial progress in treating a variety of common advanced human cancers suggests a change in approach is needed. In addition to drug resistance, the recurrence of tumors after initial tumor regression by conventional therapies is also very frequent. One potential reason for this is the failure of current therapies to target cancer stem cells. Design and development of new cancer treatments is therefore necessary to target stem cell properties; self-renewal and differentiation. If the malignancy results from a blocked ontogeny (39) then it should be possible to treat cancer by inducing differentiation (Figure 2). Over the years approaches to treat human cancers with “differentiation” therapy have been attempted. These strategies have had variable success (134, 135). Although number

of agents has been studied to target differentiation, FDA approved all-trans-retinoic acid (ATRA) and sodium phenylbutyrate (PB) have been widely used in treating haematologic malignancies which exhibit blocks in differentiation. In the haematopoietic system, blocked differentiation occurs in acute myelogenous leukemia (AML) that is characterized by the accumulation of myeloblasts in the bone marrow. AML can be divided into 8 subclasses (AML-M0 to M7) based on the differentiation of malignant cells (136). AML-M3 has a dominant accumulation of promyelocytes and that is called acute promyelocytic leukemia (APL). APL is associated with reciprocal chromosomal translocations one of which is the fusion of retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) gene with PML gene (136). The PML-RAR $\alpha$  fusion product inhibits the RAR $\alpha$  and acts as transcriptional repressor blocking haematopoietic differentiation. Differentiation induction therapy with ATRA followed by chemotherapy has increased long-term APL-free survival of patients (136-138). ATRA binds to PML-RAR $\alpha$  fusion protein and displaces mSin-3/N-CoR/HDAC deacetylation complex that causes transcriptional repression (139). However, point mutations of the RAR $\alpha$  gene confer ATRA-resistance and this can be overcome by combining ATRA with histone-deacetylase (HDAC) inhibitors. The combination of PB with ATRA has been reported to be effective in inducing differentiation in an ATRA resistant patient (140). Retinoid resistance of breast tumors were also overcome by combination of retinoic acids with histone deacetylase inhibitors (141). All these differentiation therapies are aimed at inducing differentiation of cancer cells in general may also affect the differentiation of CSCc which would lose their ability to self-renewal. This is depicted in Figure 2.

In addition to inducing differentiation, a number of stem cell self-renewal pathways have been targeted for treatment of various human tumors. As indicated above the Hedgehog/Gli pathway activated in many human tumors and in CSCs (66). Cyclopamine is a natural steroidal alkaloid that inhibits the Hh pathway by directly binding to suppressing smooth muscle receptor (142). Recent studies demonstrated that cyclopamine inhibits the growth in cell lines and xenografts from various human malignancies including breast, prostate, pancreas, medulloblastoma, small cell lung cancer, glioma and digestive tract tumors (57, 71, 143-149). Clement et al,

demonstrated that the Hh/Gli1 pathway is required for self-renewal of CD133<sup>+</sup> glioma cancer stem cells (144). This group has also compared the effect of current chemotherapeutic agent, temozomolide (TMZ) and cyclopamine in a glioma xenograft model for inhibiting tumor growth and stem cell self-renewal. Cyclopamine was shown to be effective in inhibiting self-renewal and tumor growth when compared to TMZ (144, 150). Taken together, these results suggest that successful *in vivo* blockage of the Hh pathway in tumors with increased Hh signaling may be an effective treatment which has the potential to target CSCs.

Activation of Notch signaling depends on proteolytic activity of  $\gamma$ -secretase which cleaves the intracellular domain of Notch. Inhibitors of  $\gamma$ -secretase (GSI) have been shown to inhibit Notch signaling. This pathway is activated in Ras-transformed human cells and this activation is required for the maintenance of tumorigenesis (151). Moreover, Pece et al, showed that inhibition of the Notch pathway in breast tumors with increased Notch activity can reduce the tumor growth (152). Furthermore the treatment of embryonal brain tumors with GSI-18,  $\gamma$ -secretase inhibitor not only slowed tumor growth but also blocked Notch signaling and resulted in a decreases in the stem cell population (79).

Although the mechanism is not clear, initial studies have suggested that non-steroidal anti-inflammatory drugs (NSAID) are effective in prevention of intestinal tumorigenesis in the FAP animal model (153). NSAID sulindac was shown to reduce both size and number of colorectal tumors in human FAP patients (154). Furthermore, He et al, demonstrated that NSAID sulindac and indomethacin mimic the action of APC by downregulating the transcriptional activity of the PPAR family of nuclear receptor proteins (PPAR) (155) suggesting that it inhibits the downstream targets of Wnt pathway. NSAID also inhibits the expression of COX-2 which is one of the Wnt target gene and elevated in human colorectal tumors (156). Several ongoing studies are aimed at directly targeting the Wnt/ $\beta$ -catenin complex utilizing the neutralizing antibodies or a small molecule inhibitors. A recent highthroughput screen identified a number of compounds that inhibit the TCF4/ $\beta$ -catenin complex in a reporter assay system (157). This may have potential implications for variety of human tumors with an activated Wnt/ $\beta$ -catenin pathway.

Bone morphogenic proteins (BMP) play important role in a variety of early developmental processes such as the induction of neurogenesis in neural crest stem cells and smooth muscle differentiation (158). Delivery of BMP4 *in vivo* produces a significant reduction in the stem-like, tumor-initiating precursors of human glioblastomas (GBMs). This effectively blocks tumour growth and associated mortality that occur in 100% of mice after intracerebral grafting of human GBM cells indicating a tumor suppressor activity of BMPs by mediating the stem cell self-renewal (159). Moreover the transient *in vitro* exposure to BMP4 abolishes the capacity of transplanted GBM cells to establish intracerebral GBMs (159).

The need to design molecularly targeted therapeutics for tumors based on their molecular diversity has long been recognized. An example of such targeted therapies is the use of Herceptin (Trastuzumab) in HER-2 amplified breast tumors. Despite the success of this therapy, a fraction of patients with HER-2 amplification do not respond to Herceptin and studies suggest that mutation or allelic loss of PTEN may contribute to Herceptin resistance. A recent study by Nagata et al, reported that reconstruction of PTEN in ErbB2-amplified breast cancer cell lines sensitizes these cells to herceptin treatment (109). As discussed earlier PTEN is required for appropriate stem cell self-renewal and deletion of PTEN leads to expansion of stem cell population in the haematopoietic system and prostate. Therefore the requirement of PTEN for proper action of Herceptin suggests that aberrant self-renewal due to lack of PTEN may contribute to Herceptin resistance. In the haematopoietic system, normal HSC maintenance depends on PTEN and this was mediated by mTOR. Yilmaz et al, reported that conditional deletion of PTEN in HSCs generated transplantable leukemias within weeks (113). Treatment of these leukemias with Rapamycin not only depleted leukemia-initiating cells but also restored normal HSC function (113). This demonstrates that in this system Rapamycin can selectively target the generation and maintenance of leukemia initiating cells and moreover restoring the normal HSCs. This study has important clinical implications since it suggests the feasibility of designing therapeutic approach to selectively target CSC while sparing the normal stem cell counterpart. Derivatives of Rapamycin are also been used in a number of ongoing clinical trials following the promising *in vitro* results. The cell cycle inhibitor-

779 (CCI-779) is a rapamycin ester and was shown to be effective in a selective breast cancer cell lines with increased Akt activity (160). Frost et al, reported an antitumor responses of CCI-779 in a xenograft model of melanoma and that these antitumor responses were associated with induced apoptosis and decreased proliferation and angiogenesis (161). Data from currently ongoing clinical trials of endocrine therapies with mTOR inhibitors, CCI-779 or RAD001 will be invaluable in designing molecularly targeted therapies that may target CSCs.

The tumor suppressor gene *TP53* appears to have critical role in tumorigenesis and stem cell self-renewal. Not surprisingly, *TP53* mutations occur in approximately 50% of human solid tumors and inactivation of wild type protein by the components of upstream pathways are also frequent. Small molecule inhibitors (Nutlins, Spirooxindoles) of mdm2-p53 interaction have been developed to restore p53 function in patients with wild type p53 (162, 163). The clinical use of Nutlins selectively enhanced the cytotoxicity of chemotherapeutic agents in AML blasts but not in normal haematopoietic progenitors raising the hope for the design of tailored molecular therapies. Effective treatment of AML requires elimination of leukemic stem cells that have the ability to initiate and maintain the clonal hierarchy. Although these leukemic stem cells are homologous to normal HSCs, they show aberrant expression of cell surface proteins. Of such proteins, CD44 is a transmembrane glycoprotein with many variant isoforms due to alternative splicing. Increased expression of certain CD44 variants in AML has been well documented (164). CD44 mediates interactions between cell-cell and cell-extracellular matrix through binding to its ligand hyaluronan. Jin et al, recently explored the possibility of targeting the CD44 by activating monoclonal antibody (H90). Administration of H90 to immune-deficient mice transplanted with human AML significantly reduced leukemic repopulation (165). Furthermore, the absence of leukemia in serially transplanted mice and the dramatic decrease in the number of CD34<sup>+</sup>CD38<sup>-</sup> cells suggested that leukemia initiating stem cells were targeted (165). The fact that CD44 is also expressed on a variety of CSCs including breast, colon, prostate, head and neck and pancreas suggest the feasibility that a similar approach may prove effective in treating these malignancies.

Taken together, these studies support the feasibility of selectively targeting the cancer stem cell population. Although the signaling networks that control the fate of stem cells are complex and the underlying mechanisms of action for number of candidate inhibitors are elusive. As illustrated in Figure 2, recent evidence indicates the feasibility of selective targeting of these pathways. These therapeutic strategies aimed at molecular targets which induce differentiation or death of CSCs may lead to more effective cancer therapies (Figure2).

### **Conclusion:**

This is increasing evidence that a variety of human cancers may be driven by a subset of cells termed “cancer stem cells” which retain properties of their normal stem cells counterparts. These properties include self-renewal which drives tumorigenesis and differentiation which generates the cellular heterogeneity constituting the bulk of tumors. *In vitro* and animal model studies have implicated a number of signaling pathways involved in the regulation of these cancer stem cells (Figure 1). This has facilitated the generation of therapeutic agents designed to target cancer stem cell specific pathways. Clearly, more research is needed to better identify stem cell markers and pathways responsible for maintaining this cell population. However, promising preliminary results from both *in vitro* and animal studies suggest the feasibility of selectively targeting cancer stem cells. A number of therapeutic trials based on these concepts are now entering the clinic. These therapies have the potential to significantly improve the effectiveness of cancer therapies.

### *Figure Legends:*

Figure 1. Potential therapeutic interventions involving the PTEN and Wnt pathways. PTEN activates PI3-K/Akt pathway via dephosphorylation of PI(3,4,5)P3. While PTEN activated Akt phosphorylates and activates target proteins such as mTOR, p70S6K

and S6. Activated Akt inhibits GSK3 that results in disruption of  $\beta$ -Catenin-GSK3 complex leading to activation of  $\beta$ -Catenin. A strong association of PTEN deletion and poor prognosis in many human tumors makes components of PTEN pathway attractive drug targets. Sites for potential therapeutic intervention are indicated.

Figure 2. Comparative model of conventional and alternative models of cancer stem cell (CSC) therapies. Conventional therapies fail to effectively treat advanced and metastatic tumors. The CSC hypothesis offers an alternative model suggesting the development of therapies which can target the rare cancer stem cell population. Preliminary studies suggested that the CSCs can be targeted either by directly targeting self-renewal pathways or by inducing terminal differentiation which will result in depletion of CSCs with self-renewal.

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